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Recombinant DNA debate three years on

THREE years this week, the recombinant DNA debate burst into the public spotlight with the publication in *Nature* and *Science* of an appeal for scientists worldwide to defer some types of gene-splicing experiments until their potential public health hazards had been examined. That appeal touched off an extraordinary series of regulatory battles in the United States, and the safety rules governing recombinant DNA experiments have yet to be put into final form. In Europe, meanwhile, most countries have adopted regulatory machinery with very little public interest being expressed in the issues.

In the United States, the matter now rests squarely with the US Congress, with separate bills to regulate recombinant DNA experiments working their way slowly through the Senate and the House of Representatives. The bills have become the focus of a fierce lobbying effort by scientists, and charges of 'Lysenkoism' and political control of basic research are reverberating around Capitol Hill. Though the charges are frequently exaggerated, there is indeed cause for alarm over some aspects of the pending legislation.

The facts are as follows. Last year, the National Institutes of Health (NIH) published a set of guidelines governing recombinant DNA research supported by government funds. The guidelines do not cover research sponsored by industry, however, and they are not backed by inspection or enforcement machinery. Legislation is required to correct those deficiencies, and thus Congress has every reason to be involved in the process. Instead of simply writing the NIH guidelines into legally enforceable regulations, however, Congress is likely to establish complex machinery which could introduce vast amounts of red tape into the support of genetic research. Hence the agitation by scientists.

The agitation is given extra force by the fact that information generated during the past three years indicates that the potential hazards associated with gene-splicing experiments may be more remote than once believed. For example, a special meeting of scientists and health experts, convened by NIH earlier this month, concluded that there is virtually no chance that recombinant DNA experiments could touch off an uncontrollable epidemic. Another good indication of that attitude is the following extract from a lengthy and cogent report on the ecology of *E. coli*, written earlier this year by Dr Roy Curtiss, a respected microbiologist from the University of Alabama who was among the early leaders in calling for strict controls on recombinant DNA research. "I have gradually come to the realisation that the introduction of foreign DNA into

EK1 and EK2 host-vectors offers no danger whatsoever to any human being", Curtiss wrote. Thus, while the possibility of hazard seems to be receding, scientists are being faced with increasingly complex regulations.

Most of the complaints have been directed at a bill now before the Senate, written almost entirely by the staff of Senator Edward Kennedy's health subcommittee, but with Kennedy's strong support. The legislation has already been passed by the Senate Committee on Human Resources and is likely to be considered by the full Senate later this month. A separate bill is under consideration by the House Commerce Committee and is expected to be debated by the House of Representatives later this summer.

The two bills differ significantly in their approach. The Kennedy version would establish an eleven-member Presidential Commission to draft new regulations, license facilities and approve individual experiments. The House version would require the Secretary of Health, Education and Welfare to draft new regulations, but the regulations would mostly be enforced by local biohazards committees.

The problem with the Kennedy approach is that it would establish a potentially unwieldy bureaucratic arrangement which would require researchers to obtain prior approval for their experiments from a high-level commission. Such arrangements are at present reserved for such matters as the regulation of nuclear power. The House approach offers more flexibility but without any loss of vigilance. In fact, having local committees oversee implementation of regulations is likely to be more effective than vesting such responsibility in the hands of a remote body based in Washington.

So far, the Kennedy bill has been moving through the Senate with alarmingly little discussion. There was no debate at all on the central provisions of the bill at the committee meeting when the legislation was approved, and the only forum for discussion now is the floor of the Senate, which is not the best place for deciding such complex issues. By contrast, the House version was extensively debated by Congressmen during three committee meetings, and it represents a reasonable compromise between political and scientific concerns.

At least the cries of alarm which have been coming from the research community have focused attention on the shortcomings in the Senate bill and there is now some chance that the House approach will ultimately prevail. Nevertheless, the scientists who have been charging 'Lysenkoism' and urging Congress to keep its nose out of basic research should be careful not to damage their case by overstating it. □



A world fit for whales?

Jon Barzdo, Wildlife Consultant to Friends of the Earth in Britain, asks whether the scientific basis for the management of whales through the International Whaling Commission is no more than a facade for the commercial interests of its member countries

WHEN the International Convention for the Regulation of Whaling established the International Whaling Commission (IWC) in 1946 to 'safeguard for future generations the great natural resources represented by the whale stocks' it was naturally heralded as a great advance in the conservation of whales. Indeed it was an advance, as there had been no body controlling the fishery of whales until that time; gray and right whales were given immediate protection, so low were their numbers.

The management system initially used by the IWC classified whales by the blue whale unit (BWu)—a measure of the amount of oil they produced. (1 BWu = 1 blue or 2 fin or 2½ humpback or sei whales.) Thus the system was mathematically rather than biologically based. During its use, blue and humpback whales were fished to commercial extinction. Having reached 6% and 7% of their respective initial populations both species are now protected. In 1963 a Scientific Committee of the IWC recommended the abolition of the BWu. It was abolished nine years later.

Quotas were then set for separate species in different areas, and the maximum sustainable yield (MSY) concept, introduced as the basis for management in 1974 under the 'new management procedure', upheld this system. The principle is to harvest the whales at a level (MSY level) which allows the greatest number of animals to be taken while maintaining a steady population. This is estimated to be about 50% of original population. The intention is to maintain a balance through a higher birth rate and greater availability of food. But this does not allow for competition with other species for krill. Populations of fur and crabeater seal and of penguins both appear to have flourished with the depletion of whale stocks; and we do not know the consequences of man's currently experimental harvesting of krill.

Nor does MSY allow for fluctuations in whale population, or changes in social structure through whale harvesting, or variations in the physical and biological environment (temperature, weather, food supply and so on). Discussing risks associated with different management strategies in the harvesting of baleen whales, J. R. Beddington has shown that as a population approaches MSY level it becomes increasingly unstable, increasingly vulnerable to deleterious perturbation.

Under the 'new management procedure' using MSY, over-harvesting has been such that the IWC has had cause to set zero quotas for fin and Bryde's whales in the southern hemisphere and for fin and sei whales in the north Pacific. For at least five years, and in spite of annual reductions in overall catch quotas, the whalers have consistently failed to reach their quota. Ostensibly as a concession to whaling countries, limits for some species have been regularly set above the previous year's catch level. Not surprisingly, these were never attained. In 1972 the overall quota was 38,600 whales; last year it was set at 27,939. Where do we go from here?

The IWC met in Canberra last month. It made the largest cut in quotas in its 31-year history, a reduction of 10,000 from 1976. The cuts reflect the excessively high quotas of previous years. For minke whales the southern

hemisphere reduction of over 300 to 5,690 is a result of the profound lack of data—a problem across the range, and one that increases the difficulty of estimating MSY.

The sperm whale is the most heavily taken species. The world catch limit is 7,356 although it was 23,000 only three years ago, and males have this year been offered complete protection in the north Pacific, though this limit may be revised in November. Britain imports some 8½ million kg of sperm oil (from the creature's head) annually for use in soft fashion leathers and some gear oils. The cost of the oil has been rising fast, not least because the large whales have already been fished out, and the price can reasonably be expected to soar. The leather industry might even start to use some of the range of alternatives that are available.

In 1972 the UN Conference on the Environment in Stockholm called for a 10-year moratorium on commercial whaling. The IWC rejected the call, which is supported by Friends of the Earth (FoE), the International Union for the Conservation of Nature and other conservation bodies. It would allow time for stocks to start recovering, for research into the ecology and populations of whales, and for research into a more efficient and less cruel method of killing whales than that used now.

The moratorium must go on the agenda of next year's IWC meeting. But even if it was approved it is possible that it would not be enforced. For any of the IWC's 16 member nations may legally opt out of any decision of a commission meeting within 90 days; the opting-out procedure is well used and demonstrative of the weak nature of the IWC. In addition, recommendations of its scientific committees have frequently been ignored, notably in 1960 over recommendations regarding blue and humpback whales, in 1962 over quotas and in several years up to 1972 over the use of the BWu. Moreover, there is not a single species whose yield has been sustained under the 'new management procedure'. Most are decidedly diminished.

The International Whaling Commission lacks control and effectiveness and is long overdue for replacement. To rectify this FoE propose the formulation of a UN body to manage the resources of the sea with regard to the complexity of that ecosystem, and for the general good rather than purely that of those with the (albeit ageing) technology to exploit it. □



Fighting over the whale:

Greenpeace vs the USSR

When two academies meet

Peter Pockley in Sydney outlines attempts by Australian science academies to encourage innovation

TWO learned academies in Australia are putting their weight behind a drive to educate the government and the public in the need for a higher level of local innovation in science and technology. The academies involved are the well-established Australian Academy of Science and the fledgling Australian Academy of Technological Sciences. Both have published booklets recently which catalogue and analyse the R&D scene in Australia and which advocate, implicitly, increased government initiative in this area of industry*.

Australian industry is frequently attacked for lack of concern for local innovation and for preferring instead to import new ideas. The number of research staff employed in companies with R&D capacity has dropped sharply, by an estimated 40% in the two years 1974-76. Sums invested in R&D by industry itself, though harder to quantify, are believed to have decreased significantly. Dollars from the government for direct support of R&D in industry, through an Industrial R&D Grants scheme for example, have levelled off. And indirect government support, for example through a tax incentive scheme on industry's own investment in R&D, has not been forthcoming.

Although the extent of overseas ownership of Australia industry has been a hot political issue in the 1970s, the accusation that companies controlled from overseas prefer to import their innovations is generalised largely from the vehicle industry; US-owned car companies reported no Australian innovations in 1970-75. UK- and US-owned chemical industry, by contrast, shows a high rate of innovation within Australia. And some of Australia's more dramatic innovations of late have come from government-financed laboratories, notably in CSIRO, which have developed a successful collaboration with local industry. The present Committee of Inquiry into CSIRO is charged with recommending ways and means of facilitating further collaboration.

Academy's Forum

The Academy of Science recognised long ago the need for closer liaison with industrialists. Ten years ago it

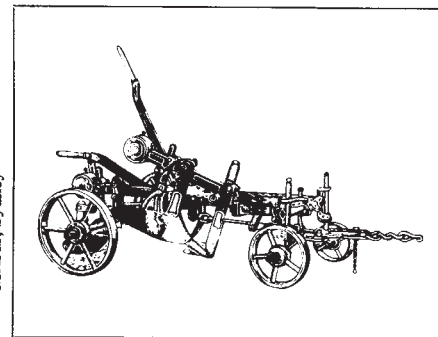
established a Science and Industry Forum with equal membership of academicians (largely basic scientists but including a few with industrial experience) and representatives of industry (businessmen as well as technologists). A pretty informal group holding weekend discussions twice yearly, the Forum has eschewed making recommendations to government but has always managed to attract responsible Ministers and Heads of Departments to its meetings.

Its recently-published set of papers on innovation, under a catchy title and with a commercial distribution and sale system, reflects the Academy's more positive, public face. In the book Sir Alan Walsh, recently knighted for services to science, avers:

To my mind there are two obstacles to invention and innovation in Australia. The first has its roots in intellectual snobbery which results in many scientists believing that any type of scientific activity which has some real bearing on industry is intellectually and socially inferior to so-called pure research . . . but probably the most serious obstacle is the widespread lack of faith in Australia's ability to generate inventions and innovations which can withstand competition from overseas. In view of Australia's record to date, this attitude is quite ridiculous . . .

The Australian industrialist is more cautious. BHP, Australia's largest locally-owned company, has a good record in Australian-based research and development. Yet its chairman, Sir Ian McLennan, believes there needs to be a "very good reason to commit support to a local invention if the related aims can be satisfactorily met from an available overseas technical development which may be purchased. As there will always be a limit to funds available to develop local innovation these are better spent in a narrower range of likely successes than used over a wide range of relatively ineffectual help . . ." His comments illustrate a growing consciousness in Australia that it is time to formulate and pursue clear national policies on the encouragement of local innovation.

The Academy of Science is a going force in Australian science, and by far the strongest. With a permanent secretariat, though modestly sized by international standards, it is able to run a range of services to science which goes far wider than keeping happy the exclusive club of its Fellowship of 183 men and two women. The visible presence of its pleasing 'igloo' building



Australia's early innovation:
the stump-jump plough

on the boundary of the Australian National University has been a significant factor in its influence. Very much a national body, it locks naturally into the bureaucratic, political and academic ethos which dominates the totally non-industrial capital of the nation.

This may have been one of the factors behind a mild blizzard around the Academy's igloo in the 1960s and early 1970s, when a gentle argument about recognition of the applied sciences built up a vortex of discontent. The storm blew around the igloo, only the mildest of breezes being felt within. One of the non-Academician activists was Dr W. A. S. Butement, a former Chief Defence Scientist. He had been leading a move by an informal group dissatisfied with the Academy's terms of admission to Fellowship; these emphasise, as the prime criterion, traditional academic achievement as perceived by peer group elections. The dissenting outsiders tried to persuade the Academy to broaden its terms to include "scientists, engineers and technologists of real worth, who are concerned with the application of science".

Constrained by the terms of its Royal charter, the Academy stuck to its chosen path. Shortly afterwards, however, it did admit to Fellowship some industrially employed scientists. As well as enjoying high standing in the hierarchy of industry, these were men with substantial publications to their name which satisfied the traditional criteria for election of basic scientists. The great majority of technologists employed in industry, universities and government departments were anonymous people by comparison. Thus began a concerted move, centred on an existing, loosely-structured forum of industrial research managers called the Australian Industrial Research Group, for a separate technological academy.

Technological Academy

The Australian Academy of Technological Sciences was formally inaugurated in February 1976 under the Presidency of Sir Ian McLennan.

* From Stump-Jump Plough to Interscan (Australian Academy of Science, PO Box 216, Civic Square, Canberra, ACT 2608).

Sir Ian McLennan, *Innovation in Australian Technology 1970-1975* (Australian Academy of Technological Sciences, Clunies Ross House, 191 Royal Parade, Parkville, Victoria 3052).

Symbolically, the function occurred in Melbourne, one of the nation's two large industrial and financial capitals; the foundation Fellowship was of 64 men and two women. The earlier rift with the Academy of Science was healed, and cooperation was ensured by the presence of 12 Fellows of the Australian Academy of Science. Criteria for election to Fellowship of the new Academy emphasise innovation, but giving to the achievements of its Fellows a collective, collegiate character is a challenge the Academy has not yet solved.

The "suitable safeguards" surrounding government or industrial documents which involve public reticence on the part of its Fellows put the applied Academy at something of a disadvantage in the public prestige stakes when compared with the freer Academy of Science. The technologists have one potential advantage in coming largely from well-heeled industries which should need little persuading to fork out financial support. But a year-and-a-half after inauguration, the new Academy is still walking along rather than jogging. In its first eight months of operation to mid-1976, it spent a mere \$4,000. It now maintains a part-time office in Melbourne, but is run on a spare-time basis by busy men. It badly needs to establish a permanent secretariat to service its present needs and future ambitions.

Australian Information Service, London

AAS's igloo

These ambitions are presently centred on the promotion of innovation in industry and government, and on the mounting of a series of annual conferences on the general theme of "Energy and Resources to 2000". The first of these, scheduled for later this year, is on "Fuel and Energy Resources", followed by "Water and Land Resources" in 1978, "Mineral Resources" in 1979, and "Marine Resources" in 1980. And of course a start has been made in publishing the first results of a review of innovative technological achievement in Australia.

One hundred years ago this year, Australia's first industrially successful

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innovation was registered—the stump-jump plough. It opened up vast tracts of otherwise useless land to cultivation. This year sees a number of internationally competitive innovations beginning to get public recognition. Successful developments, such as the compact Self-Twist Spinning Machine, the Sirotherm process for desalination by thermally regenerated resins, and the Interscan system for aircraft landing control in three dimensions, may begin to persuade government and industry alike that the two Academies are speaking economic sense when they urge more active support for locally based R&D. □

Making paper work

Christine Thomas explains the advantages in using recycled and mechanical pulp papers

FEW people are aware, when they purchase good quality writing paper in preference to rougher note paper, or choose extra-soft toilet tissue rather than lower quality crepe tissue paper, that they are contributing to increasing environmental pollution. But investigation of the demand upon resources and the polluting effects of specific processes involved in paper production highlights the considerable differences in the environmental impact of different papers. The general picture that emerges is that the higher the quality of the paper produced, the greater the resource demand and pollution load created.

Paper consists of a network of fibrillated cellulose fibres bonded together by their gelatinous surfaces.

The fibres are released from their plant structure during pulping, which for timber can be either by chemical or mechanical means. Chemical pulping methods use chemical solutions to dissolve away part of the plant structure, leaving the cellulose fibres whole and separated with minimum physical damage. Chemical pulp-based papers are considered for many purposes to be superior in quality to mechanical pulp-based papers. Mechanical pulps are generally weaker than chemical pulps and suffer from colour darkening.

Mechanical pulp papers, however, are of adequate quality for many uses, including newsprint, low grade printing papers, fibreboard, wrapping papers, and some tissues. And mechanical pulping provides a more efficient use of timber with a 90–95% yield of pulp from cut, dried wood compared to 40–65% for chemical pulping. Put

another way, where it takes on average 13 trees to produce a tonne of chemical pulp, only six would be needed for mechanical pulp. And it is not only the resource demands of chemical pulp which are greater. Its production also creates a significantly more polluting effluent, and consumes more water than does mechanical pulping (see Table 1).

Waste paper can be recycled by repulping and releasing the cellulose fibres from their existing network to allow them to be reformed into a new sheet. Naturally the substitution of recycled papers for those containing virgin pulp will result in a reduction in demand for wood pulp, and will lessen the problems of solid waste disposal. In addition, however, it will result in a reduction of energy demand, of the order of 50% of the energy required for the wood pulping process. It may also lead to less overall pollution, and a saving in water (see Table 2).

Recycled papers themselves vary considerably. Because the fibres have previously been prepared for paper-making, the recycling process in theory is simpler than pulping wood. In practice, though, there are complications introduced by the presence of ink and

contraries in the waste paper and by fibre damage due to repeated pulping. The highest grades of waste paper will produce high quality recycled papers with little or no upgrading and will create little environmental impact.

Most waste paper potentially available for recycling, however, requires some upgrading, and here the final product's quality becomes particularly relevant to the environmental impact. A bright, relatively high quality paper, made from low grade waste paper and requiring the fibres to be deinked, will consume more energy and water and will create more pollution than a lower quality paper produced from the same waste furnish. Hence, although an increase in recycling will reduce the overall environmental impact of paper manufacture, this reduction will be smaller if present high quality standards are maintained.

Encouraging recycling

How might attitudes towards paper quality be changed to encourage preferential use of recycled and mechanical pulp papers? The paper industry's resistance to using recycled fibres in some products is partly due to the resultant reduction in quality, which they maintain would not be acceptable to consumers. Whether the consumer or the paper industry needs re-educating, the government could take a positive lead by issuing advice and directives on paper quality standards to its stationery office, to local government and to government departments. This would both encourage a change in attitudes to paper quality and stimulate demand for recycled papers, thereby helping the paper industry to use more waste paper in more of its products.

Such a move could not be expected on environmental grounds alone; but there are strong economic reasons for recycling more waste paper. In 1974 Britain consumed 8.7 million tonnes of paper and board products, of which 47% was imported. Of the remaining 4.6 million tonnes produced in Britain, 45% of the furnish was from imported wood pulp. These pulp and paper imports cost Britain over £1,000 million in that year.

The British paper and board industry's heavy reliance on imported wood pulp puts it at a considerable competitive disadvantage to integrated pulp and paper industries abroad, and this has resulted in increasing dependence on imports of finished goods. In 1960, 77% of all paper and board consumed in Britain was home produced, but by 1974 this figure had dropped to 53%. The industry has suffered contraction and decline. Since 1965 over 30 mills have closed and employment

in the industry has dropped by about one-third.

In the face of economic necessity the paper industry is slowly increasing its capacity to recycle. There are considerable obstacles facing a significant increase in waste paper use. Acceptance of lower quality is only one. Plant modifications and availability of waste paper supplies are equally, if not more, important problems to overcome. The government, recognising the former problem, allocated last June £23 million for grant aid towards capital investment for increasing the use of indigenous fibres. With only £6.5 million taken up a year later both the effectiveness of the scheme and the industry's commitment to a significant

expansion of recycling are open to question.

The dominant problem in discussions of waste paper recycling concerns its collection. Potentially the waste paper is there, as Britain currently recycles only 27% of its total consumption. But instability in the market has severely weakened the collection network.

To overcome this problem, much of the effort has been focused, unsuccessfully, on stabilising the market. The tasks of building up the capacity to recycle and changing attitudes to paper quality are as important. Indeed, the strong interdependence of these three factors means they must be tackled together. □

Table 1 Average fresh water demand and discharge of pollution from pulping and papermaking in Sweden, 1970-71

	Fresh water demand (m ³ /m tonne)	BOD, ¹	Water pollution		Air pollution ² (kg/m tonne)
			Colour	Solids	
Sulphate pulping ³	250	50	210	30	20
Sulphite pulping ³	375	90	85	40	50
Mechanical pulping ⁴	50	15	—	20	0
Papermaking	130	—	—	50	0

¹Discharge of organic matter is measured by Biological Oxygen Demand (BOD₇ in kg/tonne product) and colour (platinum units in kg/tonne product). Figures include contributions from accidental discharges.

²Sulphur emissions calculated as sulphur dioxide. Sulphur emissions from oil-fired boilers not included.

³Bleached softwood.

⁴Unbleached stone groundwood.

Source: "Pollution Control in the Swedish Pulp and Paper Industry", H. Norrstrom, *Ambio*, 4, No. 2, 1975.

Table 2 Environmental impact comparison for 1,000 tonnes of pulp

	Low grade paper		Bleached pulp	
	unbleached virgin kraft pulp	recycled fibre	bleached virgin kraft pulp	deinked recycled fibres
Process water use	100 × 10 ⁶ l	42 × 10 ⁶ l	195 × 10 ⁶ l	16610 × 10 ⁶ l
Energy consumption	17 × 10 ¹² J	5 × 10 ¹² J	25 × 10 ¹² J	9 × 10 ¹² J
Air pollutants	42 t	11 t	49 t	20 t
Waterborne wastes emitted (BOD)	15 t	9 t	23 t	20 t
Waterborne wastes emitted (suspended solids)	8 t	6 t	24 t	77 t

Source: Grace and Turner, "Paper recycling and social policy", *Resources Policy*, Dec. 1976

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USA

Presidential pardon

President Carter's pruning of the White House bureaucracy has spared two science-related offices. Colin Norman reports from Washington

PRESIDENT Carter last week took the first and, he said, "for me the most difficult" step in carrying out his oft-repeated promise to slim down and streamline the federal bureaucracy. He announced a major reorganisation of the White House and the Executive Office, including the abolition of seven offices and the eventual elimination of about 250 jobs, a 15% reduction from present staff levels. In spite of early indications that the fledgling Office of Science and Technology Policy (OSTP) and the Council on Environmental Quality (CEQ) would be among the casualties, both have been spared. They will, however, be required to play their part in the weight reduction programme, and they will lose a few responsibilities.

The overall plan is being billed as a move away from the 'Imperial Presidency' and an attempt to strengthen the role of Cabinet members in formulating Administration policy. Declaring himself "very much opposed" to having the White House in total control of government agencies, with reference to the Nixon White House left unstated but clearly implied, Carter underlined the political importance he attaches to the reorganisation by announcing the plan person-

ally at a packed press conference.

In fact, the offices slated for extinction had mostly been grafted on to the White House by Congress, and their elimination will not be too fiercely resisted. They include the Office of Drug Abuse Policy, the Council on International Economic Policy, the Office of Telecommunications Policy, and the Federal Property Council. The real power centres, such as the National Security Council, the Office of Management and Budget and the Council of Economic Advisers have emerged relatively unscathed. It should also be noted that the White House and Executive Office staff has in fact grown during the first five months of the Carter Administration, from 1,655 to 1,712; the proposed reorganisation will slim it down to 1,459.

As for OSTP, it has come out of the reorganisation in a surprisingly strong position. Though the plan would reduce OSTP's staff from the 32 authorised by Congress to 22, the office would also lose some time-consuming functions, such as the preparation of an annual report on the state of science and technology in the United States. Moreover, since OSTP is still getting under way, it had not filled all of its allotted positions; the staff 'cut' will in fact hold the office to about its present size.

Frank Press, President Carter's science adviser and Director of OSTP, told *Nature* last week that he thinks the plan "looks very good", noting that he has retained all of the central functions

of the science adviser's office.

The plan would do away with the President's Committee on Science and Technology, a panel established by the legislation which set up OSTP. The committee was supposed to conduct a two-year review of federal support for science and technology, and recommend changes in the government bureaucracy dealing with research and development. Since Carter has a team of his own looking at government reorganisation, the committee's responsibilities will be taken over by that team and OSTP will be consulted. Committee members were in fact appointed late in the Ford Administration but they have never been reappointed by President Carter, and thus the committee had already informally been scrapped.

Two other important science and technology committees will also have their responsibilities changed under the plan. They are the Federal Coordinating Council for Science, Engineering and Technology (FCCSET), which consists of government officials from agencies which have responsibility for R&D, and the Intergovernmental Science, Engineering and Technology Advisory Panel, a committee chaired by the science adviser which is designed to provide a link between federal R&D programmes and state and local officials. FCCSET will become a sub-cabinet working group, chaired by the President's science adviser, and it will retain its responsibility for coordinating federal research efforts which span several agencies. The fate of the inter-governmental panel is still being worked out, but Press said last week

Debating the neutron bomb

AFTER a long and at times bitter debate, the Senate last week voted to approve production funds for the so-called neutron bomb—a small nuclear weapon designed to emit withering amounts of radiation but relatively low levels of heat and blast. The outcome of the debate was assured earlier in the week, when President Carter personally asked Congress to approve the funds. The House has yet to act on the matter, however, and Carter has also said that he will not decide whether to go ahead with production of the weapon until he has given it further study, but last week's vote is regarded as the crucial test.

The vote was 58 to 38, with several Senators who had originally expressed opposition to the weapon voting for it after Carter had made his feelings known. The Senate later approved an amendment, however, that would give Congress 45 days to pass a resolution disapproving production of the weapons if Carter eventually decides to go ahead

with them.

Perhaps the most surprising aspect of the debate about the weapons is that it nearly did not take place at all. The bill containing funds for the production of 'enhanced radiation' weapons, as warheads for the Lance short-range missile, was winding its way through Congress and had progressed a long way before the matter came to public attention. Even President Carter admitted during his press conference last week that he didn't know that the funds were in the bill until he read about it in the newspapers.

The weapons are believed to be small fission-fusion devices which would be exploded at relatively high altitudes over enemy troops. The blast area would be limited to a small region immediately beneath the detonation, but neutrons and gamma rays emitted by the explosion would be distributed over a wider area. The idea, as proponents of the bomb have been quick to point out, is that the

device would kill people while leaving most of the buildings in the area standing. But it is an extension of past weapons design, rather than a new weapon.

Proponents of the weapon have pointed out that since it would not cause massive property destruction or contaminate large areas with radioactive fallout, it would provide a more useful and credible battlefield weapon than the present, more destructive, nuclear devices. And that is precisely why the weapon has stirred so much opposition—opponents point out that it would lower the nuclear threshold by blurring the distinction between nuclear and conventional warfare. Herbert Scoville, a former top official in the Central Intelligence Agency, wrote in the *New York Times* last week, for example, that "our security depends on strengthening, not breaking, the barrier between nuclear and conventional conflicts".

Colin Norman

that its functions will be retained in some form.

At one stage, it was widely rumoured that the science adviser would be made part of a policy planning unit in the White House and that OSTP would be scrapped as a separate entity. Such a plan was in fact under consideration by the team which planned the reorganisation, but the team changed its mind and recommended that OSTP be retained. "They came to us as disbelievers but walked away believers", one OSTP official remarked last week.

Though the change of mind may have been brought about by the quality of OSTP's contributions to Presidential decision-making (the explanation offered by OSTP officials), an equally important factor is that OSTP has powerful support from the scientific community and from some influential members of Congress. When President Nixon scrapped the former Office of Science Policy, his action was greeted with a cry of alarm from a number of prominent scientists, including a committee of the National Academy of Sciences. A similar outpouring of wrath would have been certain if Carter had tried to eliminate OSTP. He would also have faced resistance from Congress—which would have to approve such a plan—and in particular from Senator Edward Kennedy, who was the chief sponsor of the legislation which established OSTP.

Similar considerations also led to the reprieve of the Council on Environmental Quality (CEQ), an agency in the Executive Office which is required to monitor compliance by other government agencies with the National Environmental Policy Act, and to provide advice to the President on long-term environmental issues. The reorganisation team was seriously considering scrapping CEQ or at least stripping it of some of its major responsibilities, but when those possibilities were leaked early in July, a hue and cry arose from environmentalists. CEQ has long been regarded as a powerful counterweight to federal agencies which conduct large public works or energy programmes likely to affect the environment, and potential demise would have been regarded as a serious blow to the environmental movement, many of whose leaders were quick to remind Carter that they had helped elect him.

In the end, CEQ will be retained, though its staff will be reduced from 40 to 32, and it will continue to monitor environmental impact statements filed by other government agencies.

By preserving CEQ and OSTP, the reorganisation plan has by-passed two potential sources of trouble in Congress, and the plan should be approved without too much of a fight. □

Breeder: far from dead?

PRESIDENT Carter's plans to deter the use of plutonium as a reactor fuel worldwide received a setback last week when the Senate voted to keep the controversial Clinch River Breeder Reactor alive. The move, which came after several hours of intense debate, will not necessarily derail Carter's policy completely, however, for the Senate at least agreed that the project should be deferred for a year, and the House of Representatives has yet to act on the matter. Nevertheless, the vote is being regarded by anti-nuclear groups as a serious blow.

The Clinch River plant is intended to be a demonstration liquid metal fast breeder reactor, designed to test the commercial feasibility of the fast breeder design. Once expected to cost less than \$1,000 million and to be in operation by the late 1970s, its estimated cost has soared to more than \$2,000 million and its expected completion date has slipped to at least 1983.

In April, as part of his nuclear non-proliferation policy, Carter proposed that the plant be cancelled and that the entire fast breeder reactor programme should be turned into a long-term R&D effort. Since the chief purpose of the Clinch River plant was to hasten the commercial introduction of fast breeder reactors, Carter argued that it no longer fits in with his nuclear strategy. The Administration's plan essentially rests on the assumption that the nuclear power programme will grow much more slowly than once anticipated; uranium supplies will thus be adequate to fuel light water reactors for several decades, and breeder reactors will not be required to augment fuel supplies by producing plutonium.

The move to downgrade the breeder reactor programme was coupled with another move away from the use of plutonium in the United States. Carter also announced that the United States would defer indefinitely the reprocessing of spent reactor fuel to re-

cover plutonium and recycle it as a reactor fuel. The announcements were designed to bolster the Administration's attempts to dissuade other countries from entering the plutonium economy, the argument being that the United States could not credibly argue against the use of plutonium without renouncing such use itself.

Although those assumptions were swiftly and sharply challenged by the nuclear industry, until recently it seemed that Carter's plan would be approved by Congress. Fierce lobbying by the nuclear industry and its supporters has, however, produced results.

The first indication that the Clinch River plant may be reprieved came last month when the House Committee on Science and Technology voted to authorise the full \$150 million for the project next year, an amount which would allow construction to begin and the plant to be put back on schedule. The House has yet to act on the committee's recommendation, however.

The next move came in the Senate Committee on Energy and Natural Resources, when supporters of the project, led by Senators Frank Church and Henry Jackson, also proposed that the Clinch River plant should be given \$150 million. The committee split 8-8 on the proposal. Church and Jackson then proposed a compromise measure: \$75 million should be budgeted for the plant, but construction should be deferred for a year. The rationale, essentially, was that the Carter Administration should use the year's delay to try to persuade other countries not to build their own breeder reactors; if the persuasion failed, the Clinch River project should be given the green light. The committee again split 8-8 on the proposal. Last week, however, after two days of debate, the Senate approved the compromise Church-Jackson proposal by a vote of 49 to 38.

Attention is now being focused on the House, which is expected to take up the matter next week. Though the Science and Technology Committee has proposed giving the Clinch River plant the full \$150 million, a compromise proposal along the lines of the Church-Jackson amendment is expected to be offered. Lobbying on both sides is fierce, but at this stage few people are willing to predict the outcome.

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Camera Press

Carter: breeder setback

Colin Norman

EUROPE

Ariane's price

If evidence is needed that the problems of international cooperation in science are more often political than scientific, the experience of the European Space Agency (ESA) is worth considering. Right now it is in the middle of its latest lurch towards compromise.

The immediate problem is money—about 7.7 million units of account (mua) of it (1 ua=£0.622). That is the amount needed to cover the extra costs imposed by the decision to transfer the launch of the scientific satellite Exosat from a US Thor Delta rocket to ESA's own heavier Ariane launcher.

The decision was reached when ESA's Council met at the end of last month, and is frankly admitted to have been political. The original plan was for a Delta launch, and tenders for the spacecraft were submitted on that basis. But an Ariane launch was always possible, and Ariane is expected to have completed four development flights (three of them with payloads) by 1980-81, when Exosat's mission of measuring cosmic X-ray sources is due to begin.

With so much money invested in Ariane and a flight available anyway, not to use it for Exosat would, it was felt, have reflected a lack of confidence. Recent unfortunate experiences with the Delta rocket, however—most spectacularly with Geos—have apparently had little influence.

When the change came, the decision was not unanimous. Ariane being a French project, France was its strongest supporter. Britain alone voted against, both in the Council and earlier in ESA's Science Programme Committee (SPC).

ESA's science advisory committee, which unlike the SPC and Council is not an executive body with national delegates, also resisted the idea. Its membership of six scientists considered the matter at the end of last year and expressed reservations about both the delay and the risk involved in an Ariane launch.

The worries are straightforward. The scientists want the satellite reliably placed in orbit as soon as possible. Estimates of the likely hold-up vary from three months to a year; it could be longer if Ariane itself goes awry, but if that happens it's probably back to the Delta. One scientist describes any delay at all as "very serious".

Meanwhile, ESA's Council meets again next week, to decide where the extra money will come from. The SPC says it can accept only 4 mua of the total. Britain objects to using funds already allocated for science on a launcher. Delays on Aerosat may ameliorate the problem, but a launch of a second Geos, at a cost of 14 mua, must also be considered. A decision on that is due in September. The options the Council has are not unacceptably limited, and some compromise seems possible. If so, it could be a prelude to something bigger later on if and when ESA's whole programme comes up for discussion.

Chris Sherwell

USSR

Messages from without

Vera Rich describes recent developments involving Soviet dissidents, and speaks to Mark Azbel

FORTY Western nuclear physicists attending the Tenth International Accelerator Conference in Dubna last week sent a message of sympathy to Dr Yurii Orlov, the physicist and dissident who since last February has been held incommunicado in the Lefortovo prison in Moscow in connection with his membership of the unrecognised 'Helsinki monitoring group'.

The scientists' action, on behalf of a colleague whose concern for academic freedom has led him into an active role in the human rights movement, reflects a growing concern with the fate of dissidents like Orlov and a recognition that such protests can be a way of offering genuine help. In the past certain scientists have expressed doubts that this type of action might only exacerbate an already dangerous situation, but since the Twentieth Party Congress in 1956 the Soviet authorities have shown a greater sensitivity to world public opinion, especially when that opinion is voiced by scientists. As Dr Evgenii Levich explained last week at a dinner in Oxford in honour of his father, who was unable to obtain a visa to attend his own 60th birthday conference, "The Soviet authorities are unable to imagine that there is any group of people as important as scientists".

This special status, of course, cuts both ways. According to Professor Mark Azbel, who for the past 2½ years ran the illegal Sunday seminar for refusnik scientists in Moscow, it is particularly difficult for physicists wishing to emigrate to receive visas. "I think that this is related to the fact that the Soviet officials are backward", he says; "they still remember only one thing—that the physicists were the ones who invented the nuclear weapons". For that reason, apparently, mathematicians (excluding applied mathematicians) generally find it easier to get exit visas than others. Not that mathematicians have it easy: Anatolii Shcharanskii, who for some time acted as spokesman of the Seminar and was an active member of the Moscow Helsinki group, is now facing a treason charge.

Azbel was finally allowed to emigrate two weeks ago. He told *Nature*: "I am infinitely happy to become, at last, a free man and a scientist in my native

country (Israel). I am infinitely unhappy, however, when I think of all my many friends who are still living in a frightful situation which I know only too well". This, he said, amounted to "gradual scientific death because of the breaking off of scientific contacts, because of lack of laboratories, because of problems with publications, because of absence of conditions for scientific work, to say nothing of the destruction of the health of both the scientists and the members of their families . . . because of the constant persecutions."

Azbel says there are still more than 100 refusnik scientists known personally to him, and that the total number "might be twice as large". This figure, of course, does not include dissidents who do not wish to emigrate. "Western scientists can do a lot," says Azbel, "as the Soviet authorities understand that collaboration with Western scientists is badly needed and that, at the same time, Western scientists do not depend on political fluctuations and that therefore they can hardly be influenced". He himself is certain that it was only due to "all those thousands of people throughout the world—both known and unknown—who supported us in our fight for scientific freedom . . ." that he was finally granted a visa. He urged that each individual case of oppression of a scientist should be taken up by his colleagues.

Ironically, the Soviet Academy of Sciences adopted a resolution last February on the "moral responsibility" of scientists to help their less fortunate colleagues. This was proposed by Corresponding-Academician Veniamin Levich, who, although a refusnik, still keeps his position in the Academy. The resolution was accepted and, as Levich says, is therefore "difficult to ignore", even though it may have no effect on his personal fate. Still, while his birthday conference was taking place in Oxford, Academician A. A. Fedorov, visiting Britain as part of a "peace" delegation, made a statement which suggested that the constant barrage of protests was beginning to take effect in Levich's case. "I hope and believe," he declared, "that Levich will be able to leave the Soviet Union very soon."

● Ernest Akselrod, a psychiatrist formerly with the space programme, who was dismissed on applying for an exit visa to Israel and has since studied psychological and medical problems of refusniks, is to face trial for "parasitism" after refusing to take an alternative job.

USSR

● The Supreme Soviet recently took time out from discussing the new constitution and assenting to Mr Brezhnev's elevation to the Presidency to consider the state of the country's forests. The current Five Year Plan provides for an extensive forestry programme, including reafforestation and planting of some 12 million ha, draining of 1.5 million ha of water-logged plantations, and routine maintenance of some 230 million ha; the total cost is over 160 million rubles.

The main shortcomings, it appears, lie in the spheres of protection and 'integrated' use of resources. There are frequent cases of wide-spread fires as checks on the observance of fire safety rules and prevention work are not always all they should be. Too little attention is given to the use of scraps and firewood timber which is often left at the felling site. Some timber is lost during rafting and transport or disappears as waste. There is a basic imbalance in the use of timber; the conifer reserves of the Urals and the north are being consumed at an intensified rate, and insufficient use is being made of deciduous varieties.

To remedy deficits in the processing industry, another source of losses, a resolution was passed "On measures to improve further the conservation of forests and the rational use of timber resources". The usual wide campaign of support in the media soon followed. The Deputy Minister of Forests of Byelorussia suggested in an article that new measures may involve a major administrative change; those forests which still belong to collective farms (some 20% in Byelorussia), he said, should not be left "with the former owners", but handed over to the state forestry fund.

● It is only recently that the Soviet Union has acceded to the idea of international control of whale fisheries, and the statement of Vladimir Tveryanovich, chief specialist at the Ministry of Fisheries, marking the close of the whaling season, has something of the enthusiasm of the newly converted. Inverting the customary Soviet pride in over-fulfilled norms, Tveryanovich proudly announced that this year the Soviet whaling flotillas had reduced the total number of whales taken; nevertheless, he added, "rational use" of the catch meant that both the variety and the quality of the output of the factory ships had been substantially

improved. He also noted that scientific teams—hydrobiologists, biologists and technologists—had sailed with the flotilla; their results, he said, will allow correct estimates of whale-stocks to be made so that the whaling industry can be conducted on a more "rational" basis. There is, as always, no suggestion of a moratorium.



In the case of the dolphin, however, there is a strong Soviet commitment to a total ban on hunting, both in the Black Sea and in the Pacific. According to Aleksei Yablokov, a prominent Soviet biologist, although dolphin hunting is totally forbidden in the Soviet Union, Bulgaria and Romania, the Turks still hunt Black Sea dolphins on a large scale. He quoted "figures from foreign experts" indicating that at least 40,000 dolphins are landed from the Black Sea, and almost as many escape from the hunters only to die of wounds. Several Soviet surveys of the Black Sea dolphin population have been made during the past few years; with the present rate of catch, says Yablokov, the Black Sea dolphin will be virtually extinct by the end of the century. As for the Pacific dolphin, between hunters and pollution it may well become a disappearing species within "a couple of years" unless some action is taken.

Yablokov stressed that dolphins are not a suitable source of raw material for industry. The skins and fat are of low value, and their meat is inedible. This last statement contrasts curiously with the campaign of 1930 which claimed that dolphin meat (in the form of sausages and other delicatessen) could make a significant contribution to the Soviet food supply, and even earn foreign currency as an exotic export. According to Yablokov, the

dolphins still have a role to play in the Soviet economy. Experiments are going forward on the training and domestication of Tursiops dolphins, which tolerate life in captivity well and will breed in captivity.

Whether these somewhat exotic plans for the dolphin materialise, Yablokov's other domestication project seems more feasible. This concerns a small school of baleen whales, living near the Shantar Islands. Since the baleen whale has a relatively small range, Yablokov suggests that they might be herded in a similar manner to reindeer, with herdsman seeking out the best "pastures" for them, and protecting them from their natural predator, the killer whale.

● The discovery of a baby mammoth, preserved in the permafrost, is proving a most exciting challenge to Soviet palaeontologists. It was found by a bulldozer operator cutting peat on the banks of the Kirgili river in the Magadan goldfields. According to Academician N. A. Shilo, the head of a special commission investigating the find, it is the best-preserved specimen so far discovered. Interviewed in *Pravda*, he said the young mammoth was 115 cm long, 104 cm high, and had a trunk length of 57 cm. The body was covered with reddish-brown hair. It was, apparently, some six months old at the time of death.

● A new 'primary standard' based on a special time and frequency system came into force throughout the Soviet Union on 1 July. The latest time standard, designed at the Institute of Radio Physics and Electronics of the Academy of Sciences of the Ukrainian SSR, is based on a ruby rod maintained at 2 K which transforms electromagnetic energy into acoustic waves with a frequency of 10^{10} Hz.

At present the Soviet Union has 105 State Standards; by 1980 there should be some 140. According to V. I. Kipichenko, Head of the Metrology Board of Gosstandard (State Standard Committee), the fundamental standard of length—defined in terms of the wavelength of ^{86}Kr radiation—"is not proving fully satisfactory in present-day conditions". An intensive research programme is accordingly under way to produce a standard of length based on laser radiation which, he says, will make it possible to link the standard length to that of time.

Vera Rich

correspondence

The laetrile question

SIR,—There is no lack of “freedom to use harmless drugs”, but freedom to promote and sell worthless remedies is constrained. Laetrile (which is not harmless), is the latest and most ingeniously promoted of a long string of such money makers. Croft's first consideration (14 July, page 100) implies that because scientists have been wrong before, therefore they are wrong this time. It is a favourite ploy of laetrile promoters' modesty to compare themselves with the Wright brothers, Louis Pasteur and other shatters of precedent.

His second consideration, the “resentment of the wealth that medical doctors extract” is an example of the “principle of compensatory evil”. Apparently it is alright for doctors to make money by prescribing laetrile. His third point seems to imply that the consumption of a cyanogenic glycoside is part of the pursuit of happiness. Unfortunately, the quest sometimes ends with the use of a stomach pump.

Would Jukes turn people away from Lourdes? Alas, I might if the admission fee were \$1,500, coupled with a promise to cure cancer.

Yours faithfully,
THOMAS H. JUKES

Nutrition planning

SIR,—In his attack on the present state of nutrition planning (30 June, page 742), Donald McLaren makes some characteristically trenchant points regarding the nature of the world's food ‘problem’, but seems to be attacking the wrong target. Surely the failure of the Green Revolution, as failure it probably is, is a classic case of the non-holistic, piecemeal approach which he advocates. It was precisely because (for reasons not unconnected with that inelegant term, *agripower*) no thought was given to the economic, educational and organisational needs associated with introduction of improved crop varieties, that the increased potential yields of these varieties did not manifest themselves as improved nutrition for the mass of poor consumers.

To implement lasting improvements in nutrition, it is surely necessary to investigate not only biochemistry, but also land tenure, demography, the availability of water and fertilisers, political power-mongering and so on,

for the relevant area. Such an analysis is, by definition, holistic. As McLaren himself points out “the widespread problem (. . .) has socio-economic roots”.

I share McLaren's doubts regarding “Utopian social engineering” and “holistic day-dreaming”; similarly the systems engineering approach of the space programme is likely to be totally inappropriate in tackling nutritional problems. Despite this, a broader approach to the study of nutrition, involving economic and institutional factors, even, possibly, the occasional maze of flow diagrams, seems to be essential. This is certainly the approach adopted in our 1978 Open University course, Food Production Systems, of which McLaren's paper on ‘The great protein fiasco’ (*Lancet* 93–6 1974), forms an integral part.

Yours faithfully,
R. M. MORRIS
Open University, Milton Keynes, UK

Unemployed scientists

SIR,—I agree with Dr Klein's comments (23 June, page 665) and would like to raise a related issue. The dire problem of post-PhD employment is still to be fully debated in Britain. The number of qualified scientists in their late twenties and early thirties with no permanent job is rising fast, especially in biology. Universities seem to be passing such people over when filling posts recently, and the reasons for this are twofold: young (ie early twenties) post doctoral job applicants are cheaper to employ and, perhaps

more insidious, there is a feeling that older candidates may compete for seniority with their longer employed, colleagues of equal age.

In the long term, such practices must be harmful to the academic community. In few other countries can an individual of 23 or 24 years of age, even perhaps five years older, be regarded as intellectually mature enough to acquire a permanent post tenable for the remainder of his working life. Here in Germany for example, the normal procedure is to spend up to six years as an *Assistent* before competing for a permanent university post.

I would like to see a list of senior, non-tenured scientists drawn up for each discipline, and circulated to the relevant university departments. Any PhD would be eligible to join this list at age thirty or so. Before advertising any post, the university concerned should first consider senior scientists on the list, and should offer the job to any suitable candidate thereby found. If none are eligible, then the post could be freely advertised.

By using a system of this kind, the backlog of skilled labour would rapidly be incorporated. Post doctoral research funds would take care of younger people waiting their turn, and an accompanying drastic decrease in PhD student numbers for the next few years would help shorten the queue.

Yours faithfully,
ROBERT RANSOM
Visiting Scientist,
Institut für Biologie II,
Universität Freiburg,
West Germany



Competition 14. £10 for the most stultifying, dreary or meaningless paragraph or two on any theme or non-theme (either published or of your own invention). The second leader of *Nature* 258, page 465 (11 December, 1975) may serve as an example. Closing date: 26 August.

Competition 13 asked for a technological message to a less advanced

civilisation out in space. Jeanne Hopkins, on the staff of the *Astrophysical Journal* in Chicago, was a clear winner with the following:

‘Congratulations on inventing the wheel! Yes, we do realise that all the tedious manual labour was performed by your grad students—but the idea was yours, and that's what counts. As you have no doubt informed your Grants Committee, your invention will revolutionise warfare; however, massive Government funding will be required to bring it to its full capability. We suggest that you submit a detailed proposal to your government, outlining possible modifications and improvements, along with a target date for each. For example: “Round wheel, target date 30 years. Toothed wheel, 300 years. White sidewalls, 3,000 years.”’

news and views

Approaches to effective anti-cancer drugs

from Stephen Neidle

ONE of the most sobering guiding principles in the development of new drugs is that it is often exceedingly difficult to improve on what nature has already provided. This tenet is especially true in the case of the topical anti-cancer agents daunomycin ($R=H$) and adriamycin ($R=OH$) (Fig. 1), first isolated some years ago from cultures of *Streptomyces peucetius* by Di Marco and his associates at the National Cancer Institute, Italy. These two compounds do indeed have considerable clinical usefulness, particularly in the treatment of acute leukaemia; adriamycin has recently achieved promising results with solid tumours as well. However, apart from being cytotoxic, (as are all anti-cancer drugs), they suffer from the very serious drawback of having severe cumulative dose-dependent cardiotoxicity (Lenaz & Page *Cancer Treatment Rev.* **3**, 111; 1976). Much effort has been directed to obtaining either new derivatives, or to developing new dose regimes, that show decreases in undesirable side effects and/or increased anti-cancer activity and selectivity. Such goals are common to many areas of cancer chemotherapy; however, in the case of daunomycin and adriamycin, it is now possible, at least in principle, to take account of recent knowledge concerning their mode of action.

It is now generally accepted that these drugs have as their primary cellular target, nuclear double-stranded DNA, to which they most probably bind by an intercalative process. The consequent inhibition of both DNA and RNA synthesis is more pronounced for cancerous cells, with their higher turnover rate. Some years ago, Pigram, Fuller and Hamilton suggested a generally plausible model for the interaction of daunomycin with DNA, based on X-ray fibre diffraction data and molecular model-building. They suggested that the aromatic chromo-

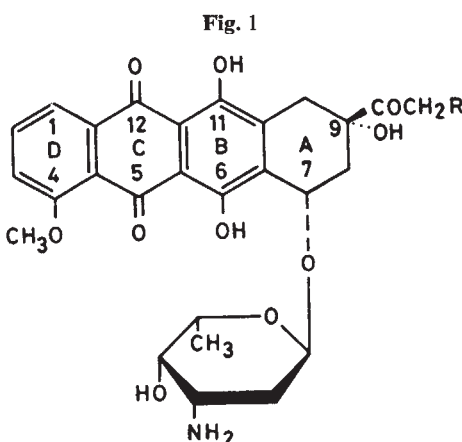
phore of the drug is intercalated in between adjacent partially unwound base pairs, and the sugar is hydrogen-bonded to the nucleic acid backbone through its amino group. The model satisfactorily explains several features of drug-DNA binding behaviour, perhaps the most striking being a very marked diminution of both binding and biological activity when the sugar stereochemistry is altered, by, say, configurational inversion (Di Marco *et al. Cancer Res.* **36**, 1962; 1976; *Biochim. biophys. Acta.* **476**, 38; 1977).

Recently there have been encouraging signs that intelligent interpretation of such a daunomycin-DNA model may well lead to more active derivatives of these drugs. (It must be borne in mind that such an approach to rational drug design tends not to take into account other factors important for activity, such as membrane permeability.) A detailed examination of the daunomycin-DNA model reveals that intercalation of the chromophore is only partial compared with simple intercalators such as proflavine; one might speculate that removal of the bulky methoxy group on the A ring would result in a molecule, that could intercalate more effectively. Di Marco and his colleagues have now synthesised, and exhaustively examined new daunomycin derivatives that lack just this group (*Biochem.*

biophys. Res. Commun. **69**, 744; 1976). The modified molecule does indeed bind to DNA somewhat better than its parent. Significantly, *in vivo* testing of these 4-demethoxy derivatives in mouse cancer systems, both of the leukaemic and solid tumour type, show that it is as effective as daunomycin itself, but at dose levels four to eight times lower (Di Marco *et al. Cancer Treatment Repts* **60**, 829; 1976).

The notion of targeting drug molecules specifically to aberrant cells, thus increasing their effectiveness, is a relatively new one. Many types of carrier for the drug can be envisaged ranging from specific antibodies to whole cells. Perhaps the classical example is the DNA-daunomycin complex proposed by Trouet (*Nature new biol.* **239**, 110; 1972) which has, perhaps unsurprisingly, proved to be of questionable value in a clinical context. However, recent reports of the successful use of liposomes (phospholipid vesicles) as versatile general carriers for, amongst others, a DNA-daunomycin complex, suggest that some of the earlier problems, such as susceptibility to endonucleases, can be overcome (Gregoriadis *Nature*, **265**, 407; 1977). Another quite distinct approach has been developed by Varga *et al.* (*Nature*, **267**, 56; 1977). They have capitalised on their earlier findings that the peptide melanotropin binds specifically to surface receptors of mouse melanoma cells; a daunomycin-melanotropin complex was also found to bind strongly to the cells *in vitro*, with a threefold molar increase in toxicity compared with the free drug. Moreover, the complex was not toxic to non-malignant mouse fibroblasts. One awaits with interest the extension of this promising approach to other cell lines, and *in vivo* systems.

The carrier concept in its various manifestations has the potential of, at least in part, overcoming the serious cardiotoxicity of daunomycin and adriamycin. The origins of this property, unique (fortunately!) to these



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drugs remain largely unknown, certainly at the molecular level, in spite of much speculation. A suggestion by Folkers (*Proc. natn. Acad. Sci. U.S.A.* **73**, 4653; 1976) that inhibition of mitochondrial coenzyme enzymes might be an important factor, has been taken up by Lown (*Biochem. biophys. Res. Commun.* **76**, 705; 1977), who suggests modifications to the anthraquinone chromophore of the drugs as a means of surmounting the cardiotoxicity. However, it does seem that such alterations would result in the undesirable effect of markedly decreasing DNA binding ability since the basic requirement for intercalation, a planar chromophore, would no longer be so well obeyed. This illustrates one of the pitfalls of this approach to molecular drug design—how optimisation of one characteristic often results in deleterious effects to others. □

Binding the *lac* repressor

from Alan D. B. Malcolm

THE *lac* repressor protein binding to *lac* operator DNA is the classic example of specific protein-DNA binding, and as such is the best characterised in terms of molecular structure. But it is still not clear why the repressor binds that particular DNA sequence so tightly and how interaction of the repressor protein with inducers such as isopropyl thiogalactoside (IPTG) completely abolishes its capacity to bind operator.

The repressor consists of four identical subunits whose primary sequence is known and studies of repressor mutants have shown that it is the N terminus which is involved in operator binding. Recently (*J. biol. Chem.* **251**, 3386; 1976) Files and Weber showed that trypsin treatment of the repressor removes the N terminal 59 amino acids from each chain leaving a tetrameric core which still binds inducer but does not bind DNA. Geisler and Weber (*Biochemistry* **16**, 938; 1977) have now found conditions for the tryptic digestion so that the intact 1-59 peptide may be isolated (albeit in a mixture with the 1-51 peptide as well). The 1-59 peptide has considerable secondary structure and can bind DNA. However it does so only weakly—with about the same dissociation constant with which

Elephants in Uganda

from Robert M. May

For the elephants of Uganda, life has got worse under General Amin. This is supported by the trends recently reported for the number of elephants in Kabalega Falls (formerly Murchison Falls) and in Rwenzori (formerly Queen Elizabeth) National Parks (Eltringham & Malpas *Oryx* **13**, 334; 1976; Eltringham *E. Afr. Wildl. J.* **15**, 19; 1977 and personal communication). The figures in the table are average numbers of elephants for wet seasons only, and are based on aerial sample counts (Eltringham *E. Afr. Wildl. J.* **10**, 299; 1972). The asterisks on the numbers for 1976 denote that they are uncorrected total counts, and therefore overestimates.

These and other African Parks were created at a time when the amount of habitat available to large animals was severely decreasing due to increases in the human population. Until the late 1920s, elephants ranged over 70% of the land area of Uganda; by 1960 the figure was 17%. As a result, large animals have tended to crowd into game reserves and National Parks, often at densities above the carrying capacity of the environment. The problem is particularly acute for elephants, whose feeding habits of uprooting and debarking trees can be very destructive. By the mid-1960s, conditions in the Murchison Falls National Park had so deteriorated that it was feared the Park would erode into a near desert, and 2,000 elephants were shot in an attempt to alleviate matters (Laws, Parker & Johnstone *E. Afr. Wildl. J.* **8**, 163; 1970). The problem of environmental degradation wrought by elephants has arisen in other East African National Parks, most notably Tsavo, and opinion is divided both about the ultimate cause and about the cure (Caughley & Goddard *E. Afr. Wildl. J.* **13**, 39; 1975; Laws *Oikos* **21**, 1; 1970). Neither the optimal density nor the optimal age structure are

well understood.

Into this vexed situation, the numbers tabulated above introduce a grim note. The precipitous decline that in 3 years has reduced the elephant population in Rwenzori by a factor of four, and in Kabalega Falls by a factor of ten, is clearly due to poaching for ivory. No attempts have been made to use the carcasses for food, nor to remove the valuable skin from the ears (an elephant hide attache case sells for \$600 to those crass enough to carry one). The elephant populations have not even enjoyed the benefits of a thinning, but rather the distribution has changed, with the animals now concentrated into relatively few pockets, where, presumably, they are safest. Eltringham and Malpas observe, moreover, that "the indiscriminate slaughter has caused a breakdown in the normally well ordered family structure: indications of a collapse in the social order are to be seen in the clumping of family units into large amorphous herds. Normally, the family is led by the matriarch which, being the oldest in the group, has large tusks and is, therefore, the poacher's prime target. With the loss of the matriarchs, the leaderless elephants have tended to congregate together, so that locally their density remains high and damage to the vegetation continues."

The experience of the past 20 years has shown that, even with the best will in the world, there are formidable ecological problems in the management of wildlife preserves. For the East African National Parks, the income generated by tourism has provided a practical motive for grappling with these management problems; with the decay of Uganda's tourist industry, it seems that mere anarchy is loosed upon the elephants.

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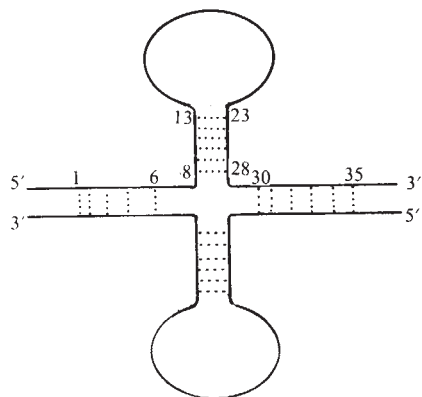
Time	Kabalega Falls (Southern sector)	Rwenzori
Average of earlier counts	8,042	2,633
September, 1973	10,187	2,864
September, 1974	4,072	1,868
September, 1975	1,061	931
September, 1976	1,346*	704*

the intact repressor binds non-operator DNA. This binding is still thought to be biologically significant however, because kinetic studies suggest that the repressor first binds to any (non-operator) sequence and then moves along the double helix until it locates the operator where it then binds 10^8

times more tightly.

The original operator region sequenced by Gilbert and Maxam consisted of the 27 nucleotides protected by the repressor from enzyme digestion. When the sequence was subsequently extended to 35 base pairs it was clear that there were four regions related by

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a twofold axis of symmetry—base pairs 1–6 are rotationally related to base pairs 30–35, and 8–13 and 23–28 are similarly related. This of course allows the possibility that the double helix be looped out to form cruciform structures as suggested over ten years ago by Gierer. Jones and Olson (*J. theor. Biol.* **64**, 323; 1977) have now suggested that if the N terminus of the protein is folded into a π helix (which has five amino acid residues per turn), one side of the helix will have tyrosines 7, 12 and 17 sandwiched between Lys 2 and Arg 22 (model studies in many laboratories have shown that such peptides interact strongly with nucleic acids) whereas the opposite face is fairly hydrophobic and would be ideal for facing in to the core of the repressor. If the operator sequence has one looped out section as shown then Jones and Olson are able to propose a perfect fit between the four subunits of the repressor and the four hydrogen bonded regions of the operator.

Unfortunately this attractive hypothesis has to face several ugly facts. The first is that a search for single-stranded regions in the operator using specific nucleases has failed to reveal any (Marians & Wu *Nature* **260**, 360; 1976). Such looped-out sequences are also out of favour in the λ operator where an expected effect on superhelical density has not been observed. Even more significantly Narang and his coworkers have been synthesising various sections of the operator and testing them for repressor binding. They showed last year (*Proc. natn. Acad. Sci. U.S.A.* **73**, 91; 1976) that the 21-nucleotide long fragment from position 8 to 28 inclusive retains full binding activity although it does not contain either of the outer palindromic sequences. Even more surprisingly, they have now shown (*Proc. natn. Acad. Sci. U.S.A.* **74**, 966; 1977) that the 17-nucleotide long region from 10 to 26 still binds although two base pairs from each of the inner palindromic sequences are now also absent. It is something of a relief to find that anything smaller than this heptadecanucleotide does lose repressor binding ability. It may be that many of the

palindromic sequences so far found at operator and promoter sites in prokaryotes are not significant after all, especially in view of the finding (Valenzuela *et al. Nature* **267**, 641; 1977; Maxam *et al. Nature*, 643; 1977) that the 5S RNA promoter in yeast has no palindromic regions at all.

Chemical syntheses of the operator region in which 5-bromouridine or

deoxyuridine have been substituted for thymidine have been reported (Yansura *et al. Nucleic Acids Res.* **4**, 723; 1977) and all the various 'mutants' tested bind repressor as well as does the wild type. It looks as though the *lac* operator is going to stretch the abilities of synthetic chemists and biophysicists to the limit before it yields the secret of its repressor binding. □

Nitrate in drinking water: health hazard unlikely

from Alan Wild

NITRATE has variously been blamed for algal blooms in lakes and rivers, for methaemoglobinaemia in young babies and most recently as a possible cause of gastric cancer. Fears have been expressed that increased use of nitrogen fertilisers on arable land will lead to a higher nitrate concentration in the water supply, and the Royal Commission on Environmental Pollution in the UK is at present considering the impact of agricultural practices on nitrate concentration in the water supply.

In the UK the Water Authorities follow the WHO standards laid down in 1970 which, for drinking water quality in general, are regarded as 'over-safe' for the UK (Pittwell *J. Institution of Water Engineers and Scientists* **31**, 214, 1977). The WHO recommended concentration of $\text{NO}_3\text{-N}$ per litre is less than 11.3 mg, 11.3–22.6 mg l^{-1} is "acceptable" and more than 22.6 mg l^{-1} is unacceptable. These standards are based on work in 1945 in which well water contaminated with coliforms (indicators of faecal pollution, another source of high nitrate levels) containing 90 and 140 mg l^{-1} of $\text{NO}_3\text{-N}$ was implicated in an illness of two infants which was diagnosed as methaemoglobinaemia (Comly *J. Am. med. Assoc.* **129**, 112; 1945). Comly recommended that well water used for infant feeding should possess a $\text{NO}_3\text{-N}$ content no higher than 10 or at most 20 mg l^{-1} . In the US and several other countries threshold standards of 10 and 20 mg l^{-1} are used. It may be noted that the WHO standards are derived from 50 and 100 $\text{mg NO}_3\text{ l}^{-1}$, and 11.3 and 22.6 may imply an accuracy that was not intended.

More recently the fear that nitrate may be implicated in gastric cancer arose from speculations that amines and nitrite react in the body to form carcinogenic nitrosamines, and that the

nitrite is formed by reduction of nitrate. There is only circumstantial evidence for this mode of formation of carcinogens (see Mirvish *Specialised Conference on Nitrogen as a Water Pollutant*, Copenhagen; 1975; Doll *Nature* **265**, 589; 1977), and it must be considered yet unproven. It should be noted that with methaemoglobinaemia and nitrosamine formation the nitrate (and nitrite) may come from food or water.

As a society we have to balance the health risk of nitrate in water (and food) against the cost of any requirement to reduce the concentration. The risk is extremely small: only one death in the United Kingdom was attributed to nitrate in water in the period 1950–75, and this followed the use of well water polluted with coliforms (Tayler *Water Treatment and Examination* **24**, 194; 1975). No cases were reported during the drought of 1976 (*House of Commons Parliamentary Debates* 22 February, 1977).

Nitrate concentrations do not often exceed 10 mg l^{-1} in public water supplies, but if they rise above the recommended standard of 11.3 mg l^{-1} the Health Authorities are automatically notified and may issue warnings to parents of young infants to use bottled water.

Concentration in river water in UK

Concentrations in river water become highest during the winter months; this is attributed to drainage from agricultural land (Slack *J. Institution of Water Engineers and Scientists* **31**, 43; 1977), and Water Authorities may then need to blend waters or withdraw some sources from use. Although analyses for 1976 have not been reported in full, it appears that there was an unusually large rise in concentration after the dry summer; for example in the two rivers Avon (Bristol) and Stour, concentrations rose to more than 11.3 mg l^{-1} in October 1976 and were over 22.6 mg l^{-1} for a short period

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(Toms Seminar Institution of Water Engineers and Scientists 29 March, 1977).

It is obviously important to know what nitrate concentrations in rivers and aquifers we can expect in the future. Extrapolating from a survey of nitrate in 18 rivers in England and Wales during 1953–67 (Tomlinson *Water Treatment Examination* 19, 277; 1970) gradual increases can be expected in some rivers but not in others. This is borne out by an increase in the annual average from 7.1–8.8 mg l⁻¹ in the river Trent and a small drop in the Severn from 3.8–3.7 mg l⁻¹ between 1971/73 and 1975/76 (data abstracted from *Water Quality* 1975/76, Severn Trent Water Authority). The response of aquifers to nitrate input occurs more slowly, but concentrations as high as 40 mg l⁻¹ have been found in the unsaturated zone above the Chalk aquifer in Hampshire (Young *et al. Water Research Centre Tech. Rpt TR* 31; 1976). In accordance with results from elsewhere, concentrations were related to ploughing of old grassland and to fertiliser use, especially the former. From the known dates of these events it was calculated that the groundwater moves through the unsaturated zone at a rate of about 1 m per year, in agreement with estimates for tritium migration, and thus higher concentrations of nitrate may reach the aquifer in 20 to 30 years' time. It is not yet known whether, before reaching the aquifer, concentrations will fall to safe levels by denitrification or by mixing with low-nitrate water.

Relative contribution from various sources

There seems to be no estimate for the United Kingdom of the relative contribution of urban, industrial and agricultural sources to nitrate in river water but an estimate from the Netherlands is that 40% of the nitrate entering watercourses is from urban sewage effluents (Cooke *MAFF Tech. Bull.* No. 32, 5; HMSO, London, 1976). Again in the Netherlands, it has been estimated that of the total 32 kg NO₃-N ha⁻¹ entering the watercourses from agricultural land, 20% comes from fertilisers, 15% from organic manures and 65% by mineralisation of soil organic matter (Cooke *MAFF Tech. Bull.* No. 32, 5; 1976). There is thus evidence here, and from the work of Young *et al.* that ploughing of old grassland is a major agricultural source of nitrate. In the UK the area (million hectares) under permanent grass decreased from an average of 7.6 in the 1930s to 4.9 in 1946, increased to 5.5 in 1955–57 and declined gradually to 4.9 in 1973 (*Agricultural Statistics for the United Kingdom*, HMSO, London),

indicating that the practice of ploughing old grassland has become uncommon. Unfortunately there is little information on the loss of nitrate from sown grassland which is a few years old, or from improved hill grazing land.

Under well manured arable crops the loss from soil and fertiliser nitrogen has been shown to give concentrations of nitrate in the land drains averaging 10 to 18 mg l⁻¹ at Rothamsted, Woburn, Saxmundham and Broom's Barn Experimental Stations (Williams *MAFF Tech. Bull.* No. 32, 174, 1976) with higher concentrations in the autumn and after a wet spring. Under heavily manured grassland the concentration in the drainage water is usually less than 10 mg l⁻¹ (Hood *MAFF Tech. Bull.* No. 32 201; 1976) and in drainage from unfertilised grassland the nitrate concentration is often less than in the rainfall (about 1 mg l⁻¹) (Cooke *MAFF Tech. Bull.* No. 32, 5; 1976). The most serious problem is therefore under arable crops, which occupy about half the farmland in England and Wales. It was estimated in 1973 that cereals, accounting for three quarters of the arable area received about 10% less nitrogen than that recommended for optimum yields (Church *MAFF Tech. Bull.* No. 32, 92; 1976). A fair conclusion seems to be that the output of nitrate into surface and ground waters from agricultural sources will increase only a little in the future although there will be differences between crop seasons determined by the weather. Nevertheless, when taken together with increased output of nitrate from sewage treatment to reduce the biological oxygen demand of the effluent, and recycling to meet an increased demand for water, the trend of nitrate concentrations seems likely to be upwards, exceeding 11.3 mg l⁻¹ more commonly in the future.

The threshold limits set for safe concentrations of nitrate in drinking water are now seen to be critical. There is no evidence to support the WHO limits and we should recognise the absence of risk to adults with concentrations of 22.6 mg l⁻¹, a requirement that Water Authorities can easily meet. With young infants it seems advisable to keep to an upper limit of 11.3 mg l⁻¹ until evidence indicates otherwise, and to supply low-nitrate water in bottles as required. An 'over-safe' limit will increase the costs of purifying drinking water and of sewage treatment quite unnecessarily, and might bring unreasonable pressure on farmers to restrict the use of fertilisers (which now produce food worth about £1,000 million per annum). At the same time there is a clear requirement to

establish from sound medical evidence the upper permissible limit especially for young infants and to improve the models used for forecasting changes in nitrate concentrations, which means identifying sources and measuring rates of denitrification in surface and ground waters. It is to be hoped that the Royal Commission on Environmental Pollution will allay the unjustified public anxiety over nitrate in drinking water and also point to ways of meeting these two requirements. □

Insulin genes into *E. coli*: first step from fantasy to fact

from Peter W. J. Rigby

ONE of the major benefits to accrue to society as a whole from the exploitation of *in vitro* DNA recombination may well be the production by industrial fermentation of medically important proteins such as antibodies and hormones, which are expensive to isolate from animal sources. The most popular scenario of this type involves the production of polypeptide hormones, particularly insulin, by isolating the relevant gene as a recombinant plasmid and forcing the expression of the inserted eukaryotic DNA within *E. coli*. A recent paper (Ullrich *et al. Science* 196, 1313; 1977) describes the first step towards this objective, the isolation of part of the DNA sequence coding for rat insulin.

This work, performed in the laboratories of William Rutter and Howard Goodman at the University of California Medical School in San Francisco, depends on the already classical approach of copying mRNA into double-stranded cDNA using reverse transcriptase and then inserting the cDNA copy into a bacterial plasmid vector. So far most work of this type has involved genes such as globin, whose mRNA can be obtained in highly purified form (Efstratiadis *et al. Cell* 7, 279; 1976). After introducing the recombinant plasmids into *E. coli* by transformation, bacterial colonies containing the desired sequence can be identified by hybridisation with an appropriate probe, generally the purified mRNA. Pure insulin mRNA is not available however, and so an indirect approach has had to be adopted. The success of this work has also depended on several ingenious technical developments which should find widespread application in recombinant DNA research.

The first problem is simply the isolation of insulin mRNA, synthesised

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only in the islets of Langerhans in the pancreas, which is a very rich source of RNase. To circumvent this problem Ullrich *et al.* used a previously described procedure to isolate islets, thus increasing the proportion of insulin-synthesising cells, and then isolated RNA by a method, developed in Rutter's laboratory, for preparing intact mRNA from sources rich in RNase.

cDNA prepared from total polyadenylated mRNA isolated in this way was characterised by gel electrophoresis both before and after digestion with the restriction endonuclease *Hae*III. Undigested cDNA showed a prominent band about 450 nucleotides in length, while the *Hae*III digest showed bands of 180 and 80 nucleotides. The strategy of the subsequent work is completely predicated on the assumption that these prominent bands are derived from insulin cDNA, which should be a major component of the total cDNA.

Double-stranded cDNA synthesised in this way is in fact a single DNA strand with a hairpin-loop structure. In the conventional strategy for cloning such cDNA the next step would be to open the loop by digestion with S1 nuclease, add homopolymeric tails and then join the tailed cDNA to a vector carrying complementary homopolymer tails. A major disadvantage of this approach is the absence of a reliable method to excise the complete insert from the recombinant plasmid. Ullrich *et al.* have overcome this problem by using chemically synthesised restriction endonuclease recognition sites as linkers. The cDNA was digested with S1 nuclease to open the loop and render it perfectly double-stranded; this blunt-ended DNA molecule was then joined at both ends to a decanucleotide containing the *Hind*III recognition sequence. Such blunt-ended joining can be performed using the DNA ligase induced by phage T4, rather than the *E. coli* enzyme which requires cohesive termini. The *Hae*III endonuclease fragments were similarly joined to the linker; in this case S1 digestion was unnecessary as *Hae*III produces blunt ends. Digestion of the products of these reactions with *Hind*III endonuclease produces DNA segments with cohesive ends, which can be joined to *Hind*III digested vector DNA, in this case the plasmid pMB9.

While such cohesive end joining is convenient and simple, it suffers from a major disadvantage in that the vector can recircularise and thus many of the bacterial colonies selected for expression of the marker function carried by the vector are found to contain merely the reconstituted vector, with no insert of foreign DNA. By treating the *Hind*III digested pMB9 DNA with alkaline phosphate to remove 5'-phosphate

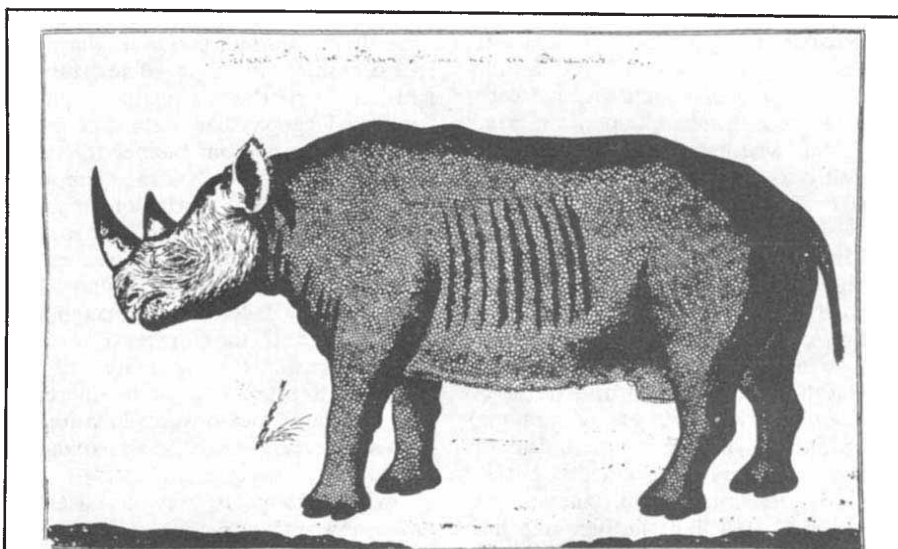


PLATE II. Gordon's second *Dicerus bicornis* figure, the head more naturally poised. (Gordon Atlas, fig. 206. Rijksmuseum, Amsterdam).

Illustration from 'Robert Jacob Gordon's original account of the African Black Rhinoceros' (Cave & Rookmaaker *J. Zool., Lond.* **182**, 137; 1977). This provides an account of the memoranda and drawings comprising a description of the African Black Rhinoceros prepared at the Cape of Good Hope in 1778 by Robert Gordon and now incorporated in the Gordon Atlas preserved in the Rijksmuseum, Amsterdam. Although 'Gordon's rhinoceros' was temporarily recognised, Gordon's material never attained independent publication and the historical and anatomical merit of this pioneer investigation went largely unrecognised.

groups Ullrich *et al.*, prevented this recircularisation. Only those plasmid molecules which have annealed to foreign DNA carrying 5'-phosphates can be covalently closed by DNA ligase and thus the background in the transformation step is drastically reduced.

Having introduced these recombinants into *E. coli* by transformation and selecting for tetracycline resistance, the marker function carried by pMB9, the next step in the experiment would normally be to screen for the desired sequence by hybridisation. As no suitable probe was available, crude DNA preparations were made from each clone and these were screened by restriction endonuclease digestion, to look for plasmids containing inserts of 450, 180 or 80 nucleotides, the sizes predicted from the characterisation of the cDNA. One clone derived from total cDNA and containing an insert of 410 nucleotides was identified in this way. By a slight variation on this procedure clones containing the 180 and 80 nucleotide *Hae*III fragments were also obtained.

Even at this stage of the experiment there was no direct evidence that the inserts corresponded to insulin-coding sequences.

The identity of the cloned cDNA was, however, established in the most convincing way possible by direct sequence analysis which showed that it coded for the known amino acid sequence of rat insulin. The immediate

product of the translation of insulin mRNA is preproinsulin, which is processed to the mature hormone by a series of proteolytic cleavages. The four plasmids isolated together cover the whole of the proinsulin coding sequence as well as part of the N-terminal pre-peptide. Unfortunately none of the clones contains sequences from the 5' untranslated region of the mRNA.

This work, demonstrating that particular nucleic acid sequences can be isolated in the absence of any screening procedure other than direct sequencing of the final recombinants, represents something of a triumph for 'brute-force' molecular biology. However, the general applicability of such methods is unclear. Not until the sequencing was quite advanced could Ullrich *et al.* have been confident that islet mRNA did not contain a major component which was not insulin mRNA. It seems likely that if this approach were used in other cases the wrong sequence would be cloned. In this regard it is particularly unfortunate that the paper contains no data bearing on the efficiency of the procedure; it would be of great interest to know how many clones were screened to obtain the four containing insulin sequences.

We can confidently expect these plasmids to be used as probes in experiments to isolate the complete insulin gene together with its flanking sequences from total rat DNA. Once

such recombinants are available it will be possible to explore approaches to forcing the expression of the insulin gene within *E. coli*. Techniques already exist to force transcription of inserted DNA, but whether such transcripts can be faithfully translated is unknown. Even if *E. coli* could be persuaded to synthesise preproinsulin there still remains the problem of processing this to the mature hormone. The use of this technology for the large scale production of insulin may well depend not only on engineering *E. coli* to express the insulin gene but on the development of *in vitro* processing systems. There clearly remains a great deal of work before one can confidently anticipate the commercial production of insulin in *E. coli*, but the first step has been taken. □

Evidence for Earth expansion?

from Peter J. Smith

THE concept of an expanding Earth has been in circulation for several decades now, but has never attracted much interest. If it were not for the historical example of continental drift, it would be tempting to assume that such indifference reflects a correct interpretation of the Earth's behaviour and that expansion is only an illusion in the minds of a handful of enthusiasts. But the temptation should be resisted, at least for the time being. Drift was once regarded as illusory by the majority of Earth scientists; but the idea was kept alive by a few fanatics until proof came at last from outside the field of geology. Geologists, convinced that continental drift was inconsistent with the existing body of geological knowledge, suddenly found their rejected theory confirmed by physicists and were forced to reinterpret their data accordingly.

Of course, it would be dangerous to over-react to history by concluding that the majority must now be wrong about Earth expansion as it would be to re-enact the sort of response which greeted Wegener's suggestion that the continents had drifted. The cases are not precisely analogous. There were serious problems with the pre-drift world view that only drift could resolve, whereas Earth expansion appears to offer no comparable advantages. On the other hand, if proof of expansion is to come at all it is most likely to come, as with drift, from the physicists. Earth scientists may feel they have no need for expansion; but if physicists could show that the gravitational constant has decreased with time, expansion would

have to be accepted and accommodated.

In the meantime, however, there are still occasional attempts to test the expansion hypothesis against purely geological-geophysical data, the latest of which comes from Steiner (*Geology* 5, 313; 1977). Steiner's idea is simple in principle. For an Earth of constant radius, the rate at which the surface area of ocean floor increases at oceanic ridges must equal the rate at which it decreases through subduction. But if the former is greater than the latter the total area of the ocean floor must *ipso facto*, increase; and the most obvious explanation of such an increase would be an expanding Earth.

Unfortunately, as previous attempts have shown, the practice of testing for expansion is invariably much more difficult than the principle. Steiner begins well enough with the map of ocean basin ages produced by Pitman *et al.* (*Geol. Soc. Amer. Map MC-6*; 1974), from which he measures directly the area of floor produced in the three major oceans during each geological epoch and period from the Jurassic to the Holocene. The first conclusion to emerge clearly is that at most times the rates of floor production vary considerably from ocean to ocean but with "a tendency toward negative correlations where a maximal rate of sea-floor spreading in one ocean basin is compensated for by a minimal rate in another". During the Paleocene, for example, the rates in the Indian and North Atlantic Oceans were high compared with those in the Pacific and South Atlantic Oceans; during the Eocene the rate in the Indian Ocean was higher than those in the Pacific and North Atlantic Oceans which were in turn higher than that in the South Atlantic Ocean; and so on.

The existence of such negative correlation indicates that sea floor spreading results from a globally coordinated process, although this no more rules out the possibility of traditional convection than it does the possibility of expansion. Slightly more favourable to expansion (though again not definitive), perhaps, is the fact that the combined rate of ocean floor production for all the oceans together has apparently increased exponentially with time since the Jurassic. Of course, this detected increase could be spurious. The ocean floor data currently available would indicate an increase even if the rate of floor production had always been constant, for the older the sea floor the greater generally is the proportion already subducted. Moreover, the picture is confused by the fact that the map produced by Pitman and his colleagues is incomplete, specifying ages for only about 66% of the deep ocean floor.

To determine the true rate of floor production since the Jurassic, Steiner has therefore had to make estimates of the spreading in unmapped areas during the past 5 Myr, extrapolate the results back in time, and make corrections for differential subduction. The increase in the rate of floor production with time remains, nevertheless, even when these procedures are complete.

As for numbers, Steiner estimates that the average rate of ocean floor production over the past 165 Myr was $2.61 \text{ km}^2 \text{ yr}^{-1}$. He also concludes that the average subduction rate over the same period was only $1.88 \text{ km}^2 \text{ yr}^{-1}$ and that this average too conceals an increase with time. However, the procedure for obtaining the latter figure is, if anything, even more involved than that necessary to determine the average spreading rate; and therein lie two difficulties. The first is the question of credibility. The greater the degree of assumption, extrapolation and 'correction' there is in calculations of this sort, the less convinced the reader will be of the validity of the result and the greater will be the opportunity for picking holes in the argument. This certainly implies no criticism of Steiner, who has done the best he can with the limited data available. In the circumstances, however, it is bound to be difficult to convince those who are predisposed not to believe in Earth expansion.

The second problem, which may only be a special case of the first, is that the more complicated the procedure and the larger the number of mathematical processes involved, the greater will be the errors on the figures obtained. Thus Steiner estimates the errors to be $\pm 15\%$ on the areal production rate and $\pm 20\%$ on the subduction rate. The obvious conclusion to be drawn from the rates themselves is that over the past 165 Myr the area of ocean floor produced was 33% greater than the area destroyed, which interpreted in terms of an expanding Earth would imply a Jurassic palaeoradius of 5,668 km, or 0.89 of the Earth's present radius. The final error on the palaeoradius, however, is $\pm 13\%$. Unfortunately, this is just large enough to allow the figures to be interpreted in terms of an Earth of constant radius even though, as Steiner points out, there is good reason for regarding 5,668 km as a maximum.

So Steiner has come up against the problem that has bedevilled most previous attempts to test the hypothesis of Earth expansion, namely, that the basic data have insufficient resolving power. Through no fault of his own, his analysis is unlikely to convert anyone to a belief in expansion, although equally it will do nothing to discourage those already converted. □

review article

Quark confinement

R. L. Jaffe*

Nucleons and other subnuclear particles seem to be composed of more fundamental objects, the quarks, but bombardment of nucleons at increasing energy has failed to liberate quarks from the interior of nucleons. This has led to the development of theories in which quarks are of necessity permanently confined. Models of the dynamics of confined quarks, such as the 'MIT bag', have unified our understanding of subnuclear phenomena.

FROM time to time in the past the 'fundamental particles' of physics have been shown to be composites of particles yet more elementary. Molecules are composed of atoms, atoms in turn of electrons and a nucleus, the nucleus in its turn by protons and neutrons. Now there is impressive evidence that protons, neutrons, and the host of short-lived particles with which they interact strongly are composed of yet more fundamental objects called quarks. Quarks, however, have not been found free in nature; and if present theories are correct they never will be: they are thought to be permanently confined to the interior of the particles they compose.

This is a radical departure from the behaviour of other more familiar composite systems. It is relatively easy to separate an atom from a molecule, an electron from an atom or a proton from a nucleus. It is thought not merely to be difficult but actually to be impossible even in principle to remove a quark from inside a proton. This idea is rather young. It has been taken seriously only since early this decade. We have no fundamental explanation of quark confinement, nor an operational description consistent with relativity and quantum mechanics—still the foundations of modern physics. Nevertheless, in the past few years a possible theory of quarks has emerged which is aesthetically attractive and at least consistent with confinement; and an operational model incorporating confinement has been successful in describing many phenomena. Before describing these advances it is necessary to outline the events which forced the extraordinary notion of permanent confinement upon us.

The problem

Today's 'elementary' particles are of two types. There are a few 'leptons'—the electron and neutrino being the most familiar—and the photon, which interact only weakly with one another and with all others. So far these particles have shown no internal structure. They seem to be simple and may be fundamental. Because of their relative simplicity they are useful as probes of the other particles, the 'hadrons', which are certainly extremely complex. Hadrons are defined by the fact that they interact very strongly with one another. The best known are the proton and neutron. The well known effects of nuclear fission and fusion are manifestations of the extremely strong forces between hadrons.

It is the hadrons which are believed to be composed of quarks. In addition to the proton and neutron and the heavier nuclei there are hundreds of known hadrons. All of these others are very short lived. Hope has been abandoned that the hadrons themselves are elementary. Even the proton is not special. It

happens to be stable only because there is no lower energy system for it to decay into. In fact the hadrons behave more like excited states of one particle, the hadron, than distinct entities.

With such a host of particles, classification becomes a major task. Hadrons, like atoms or nuclei, may be labelled by their quantum numbers. Examples familiar from atomic and nuclear physics are intrinsic angular momentum (called spin and labelled J) in units of Planck's constant $\hbar$; parity (P), which labels reflection symmetry, and takes values ± 1 ; and electromagnetic charge (Q) in units where the charge of the proton is $+1$. Quantum numbers are important to the extent that they are conserved by interactions. Charge, for example, seems to be absolutely conserved by all interactions. Hadrons carry other quantum numbers which have been painstakingly discovered over the years: 'baryon number', 'strangeness' and 'charm' are the names of ones of importance to us. Hadrons come in two broad categories: baryons and mesons, which are distinguished by their decays. All hadrons decay by emitting leptons, photons and/or other hadrons, the last of which in turn decay in the same manner. Baryons always leave a proton among their decay products. Their antiparticles, antibaryons, always decay eventually into antiprotons. Mesons either decay entirely into leptons and photons or into baryon-antibaryon pairs. To quantify all this, baryons are assigned 'baryon number' $+1$, antibaryons are assigned $B = -1$, and mesons are assigned $B = 0$. The decay patterns are equivalent to conservation of baryon number. No known interaction violates this conservation law.

The detailed significance of strangeness (S) and charm (C) need not concern us here. It is necessary only to regard them as measurable integer labels carried by hadrons, which are conserved in strong interactions but not in the weak interactions responsible for nuclear beta-decay. Quite early in the game it was observed that strange hadrons ($S \neq 0$) were systematically heavier than non-strange hadrons with otherwise identical quantum numbers. When hadrons with charm ($C \neq 0$) were discovered last year they were found to be heavier still. It is quite possible that there are other even heavier hadrons with other labels which have yet to be discovered. Quantum numbers like charge, strangeness, and charm which distinguish among mesons and among baryons are known generically as 'flavours'.

The proton and neutron have very nearly the same mass (about 938 MeV) and are quite similar in many other respects. They differ, of course, in their electromagnetic charge. All hadrons follow this pattern: they come in (small) families or 'multiplets' with very similar properties but different charges. The explanation (proposed long ago by Heisenberg¹) is that the strong interactions are to a very good approximation 'charge-blind'. This symmetry, known as 'isospin' symmetry, describes the types of families and the similar properties of members of a family but does not determine which families exist. Why the

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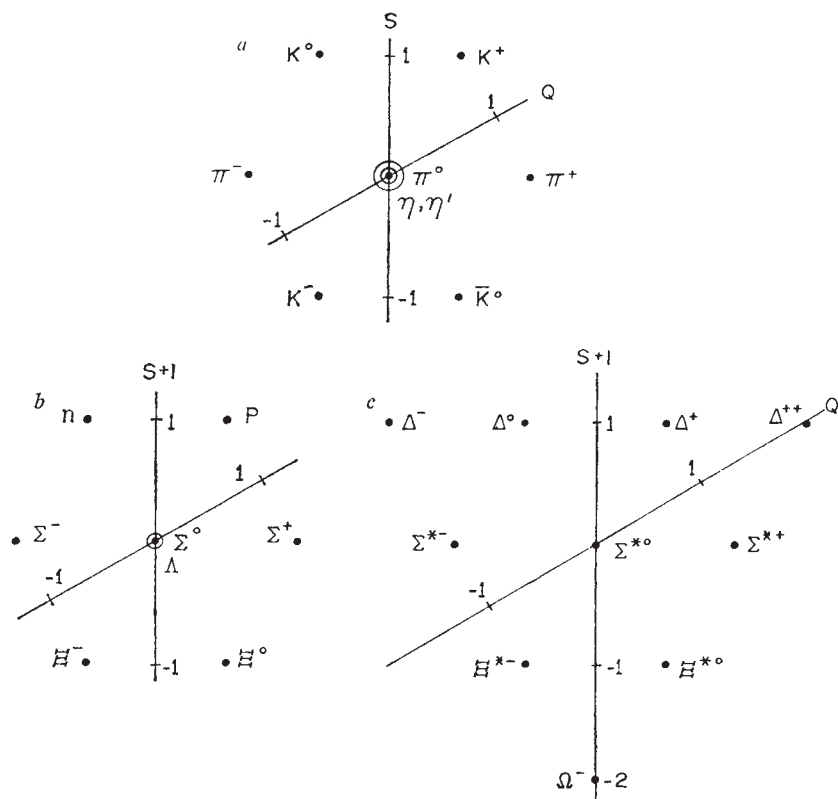


Fig. 1 Hadron multiplets: strangeness is plotted against charge using inclined axes to better display the symmetry. All hadrons in a multiplet have the same intrinsic spin and parity. Within a multiplet states with strangeness are systematically more massive. *a*, 0^- meson nonet; *b*, $\frac{1}{2}^+$ baryon octet; *c*, $\frac{3}{2}^+$ baryon decuplet.

proton-neutron form a doublet, while the π -mesons form a triplet (charges $-1, 0, +1$), and the Δ -baryons form a quadruplet (charges $-1, 0, +1, +2$) is not explained by this observation.

After the discovery of strange hadrons in the early 1950s attempts were made to incorporate strangeness into the classification scheme. The idea was to assign hadrons to families with similar properties (in particular with the same spin and parity) but with different charge and strangeness. Strange hadrons weigh somewhat more than their non-strange siblings so the strong interactions are not blind to strangeness, but they are near-sighted enough to allow us to group particles into families anyway. The correct scheme was discovered by M. Gell-Mann² and Y. Ne'eman³ in 1961. The arrangement of charges and strangeness within a multiplet and the number of states in the multiplet are closely related and are dictated by the mathematical formulation of this approximate symmetry. Some of the most prominent hadron multiplets are shown in Fig. 1. Before the advent of charm in 1976 all known mesons were found to come in multiplets of 9 (nonets) and all known baryons in groups of 1, 8 or 10 (singlets, octets or decuplets).

Still there was no explanation for this apparent multiplet structure. To borrow an analogy from atomic physics, the scheme of Gell-Mann and Ne'eman is like the periodic table of Mendeleev. It classifies, but does not explain, the properties of hadrons.

The notion of quarks was introduced in 1964 independently by Gell-Mann⁴ and Zweig⁵ to explain why baryons come only in multiplets of 1, 8 and 10, and mesons in multiplets of 9. They proposed, for the purposes of classification, to imagine that hadrons are composed of three types of more fundamental particles—quarks⁶. Baryons were to be made of three quarks

(Q^3), mesons of a quark and an antiquark ($Q\bar{Q}$). To get the hadron quantum numbers right quarks must have very odd quantum numbers themselves. If the proton is to have baryon number one, then quarks must have baryon number one-third. To give the correct electromagnetic charges for the hadrons quarks must have charges which are $2/3$ or $-1/3$ times the proton's charge. No one has ever observed a particle with fractional charge or baryon number. In 1964 the idea was so radical that most physicists preferred to view quarks as theoretical conveniences—an efficient way of summarising the observed hadrons—not as real objects expected to be observed in the laboratory.

Gell-Mann's three quarks were named *u*, *d* and *s* (for 'up', 'down', names which arose from the formalism of isospin and 'strange'). The quarks' quantum numbers are listed in Table 1. To make mesons combine any quark and antiquark. There are nine combinations with a given total spin and parity just as observed. The quark content of a sample nonet is shown in Fig. 2. Baryons are somewhat more complicated. The families of 1, 8 and 10 are shown in Fig. 3. A bit of tedious mathematics would be required to explain why only these multiplets are allowed. By combining the spins of three quarks it is possible to make multiplets with total spin $1/2$ or $3/2$. Nature, however, is more selective: the octet containing the proton and neutron has no spin $3/2$ counterpart; the spin- $3/2$ decuplet shown in Fig. 1 has no spin- $1/2$ analog. What forbids these other configurations? The answer lies in permutation symmetry. The characteristic shared by the allowed baryon configurations is that they are totally symmetric with respect to exchange of any pair of particles. Apparently, then, the quark model must be augmented by the rule that only symmetric configurations are allowed.

This raised a serious puzzle. Long ago Pauli⁷ demonstrated a

Table 1 Quantum numbers of quarks

Quark	Charge	Baryon number	Strangeness	Charm	Spin	Mass (MeV)
u (up)	$2/3$	$1/3$	0	0	$\hbar/2$	0
d (down)	$-1/3$	$1/3$	0	0	$\hbar/2$	0
s (strange)	$-1/3$	$1/3$	-1	0	$\hbar/2$	300
c (charmed)	$2/3$	$1/3$	0	1	$\hbar/2$	1500

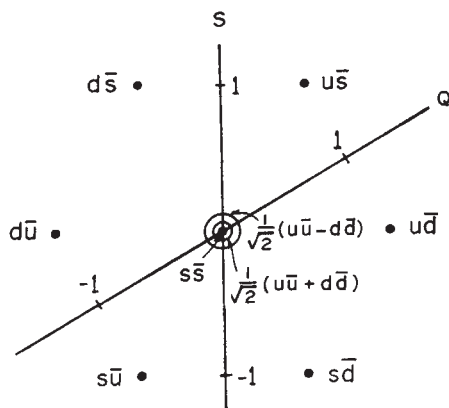
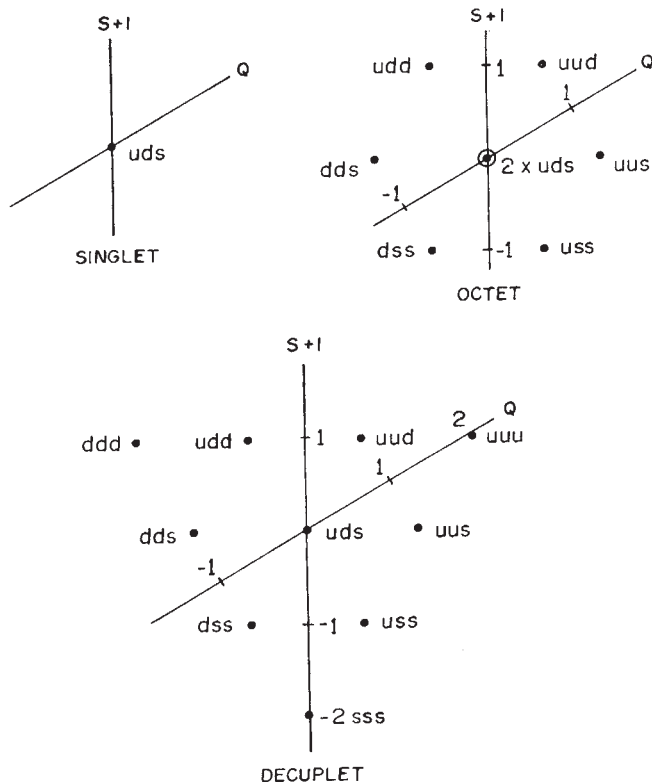


Fig. 2 The quark content of a typical meson nonet. Compare Fig. 1a.

deep connection between symmetry and intrinsic spin: particles with integer spin (in units of $\hbar$) combine with each other only in symmetric configurations while those with half-integer spin combine only in antisymmetric arrangements. Quarks seemed to violate this. Early on this seemed yet another reason not to take the quark idea too seriously. Now it seems to be an essential clue to resolving the mysterious behaviour of quarks. We shall return to it shortly.

Over the years it has proved possible to account for a great variety of phenomena by supposing hadrons to be made of quarks⁸: the magnetic moments of the proton and neutron, photo-excitation of the proton, very high energy scattering of hadrons off one another, to name only a few. The flavour conservation laws observed in strong interactions reduce to the simple rule that the strong force never changes a quark's flavour. The strangeness of a bunch of hadrons is just the number of $\bar{s}$ -antiquarks minus the number of s -quarks. Strong interactions may create or annihilate $s\bar{s}$ -pairs but the total strangeness is conserved. Weak interactions are flavour changers: in the β -decay of a neutron, for example, sd -quark changes into a u -quark.

Fig. 3 The quark content of a typical baryon singlet, octet and decuplet. Compare Fig. 1b and c.



The major problem with the quark model was thought to be the failure of attempts to observe free quarks. Originally this was explained by assuming them to be very heavy (say thousands of MeV) and to be bound by very strong forces to make much lighter hadrons like the proton. To remove a quark from a proton would require restoring the binding energy and would be difficult. This is not 'confinement' as we now understand it but just 'binding' as in atomic physics on a grander scale. This picture was difficult to reconcile with the way the model had been applied: quarks were treated as free and light (roughly 1/3 as massive as the proton) and the properties of hadrons were obtained just by adding up the properties of quarks. Interactions generally modify the properties of particles, the stronger the interaction the greater the modification. If the quarks within a proton were bound with binding energy equal to several times their rest mass it seems uncanny that the unmodified quark properties should persist.

Quarks were only 'mathematical' particles anyway. It seemed premature to worry about these dynamical puzzles until someone had actually discovered a quark. During the late 1960s many quark searches were undertaken—none was successful. Finally a series of beautiful experiments⁹ led by J. Friedman and H. Kendall of MIT and R. Taylor of SLAC begun in 1967 obtained indirect but persuasive evidence for the existence of quarks within the proton. The MIT-SLAC collaboration scattered high energy electrons off proton targets. The motion of electrons is effected by the local electromagnetic currents within the proton. Electrons do not interact strongly and are relatively structureless. Because of this they make excellent probes of hadrons. The greater the angle through which the electron scatters the shorter distances within the hadron it probes. The experiments showed that when scattered through large angles, the electrons behaved as though scattered elastically from light, spin-1/2 constituents within the proton. This behaviour is known as 'Bjorken scaling' after J. D. Bjorken of SLAC who first suggested such behaviour might be expected¹⁰. Similar phenomena are well known in other branches of physics¹¹. Consider scattering of electrons off a nucleus. At very small angles the electron most often scatters elastically from the nucleus as a whole. At larger angles the scattering is predominantly inelastic, leaving the nucleus in an excited state or fragmenting it into a few pieces. At even larger angles the electron scatters nearly elastically from the individual protons in the nucleus. For this to occur the energy transferred to the proton must be large compared with its binding energy. So the proton is knocked out of the nucleus and could be observed propagating freely after the collision. If the proton had not been independently discovered long before such experiments were performed the behaviour of the scattered electron would have been taken as exciting evidence that the nucleus is composed of weakly bound charge 1 constituents—protons. The neutrons, being electrically neutral, would have been missed.

So it was with quarks. Electron scattering from a nucleus and from a proton are compared in Fig. 4. The observation of Bjorken scaling indicates that there are more fundamental, local bits of charge within the proton. These were called 'partons' by Feynman^{12,13}. This series of experiments and subsequent similar neutrino-proton scattering studies suggest that the proton's 'parts' are quarks. In fact there is no serious alternative description of large angle lepton scattering. So hadrons are made of quarks. There might also be electrically neutral quanta (which would not show up in these experiments) to mediate the interactions between quarks. These are known generically as 'gluons', the 'glue' which holds the proton together.

It must be emphasised that the MIT-SLAC experiments showed the proton to be a rather loosely bound collection of quarks. At distances of the order tenths of a fermi within a proton (the proton's radius is about 1 fermi = 10^{-13} cm), there was little if any evidence for forces on the quarks. In short, everything was analogous to inelastic electron scattering off nuclei except that in the nuclear case protons are being knocked out of the nucleus while in our case quarks are not being knocked

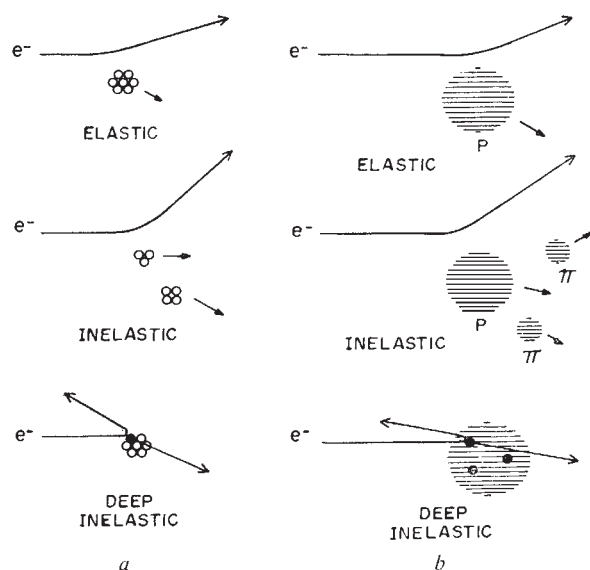


Fig. 4 A comparison of elastic and inelastic electron scattering off a nucleus (a) and a proton (b). In each case as the scattering becomes more inelastic, it becomes more sensitive to the local charge structure within the target. In case (a) when the energy transferred to the nucleus is much greater than a proton's binding energy, the electrons seem to scatter elastically from the individual protons. In case (b) the electrons eventually seem to scatter elastically from quarks within the proton.

out of the proton. Hadrons appear to be weakly interacting aggregates of light quarks which nevertheless can never be removed from the interior of the particles they compose.

In the years since the original MIT-SLAC experiments, evidence supporting the quark model has steadily accumulated. During the early 1970s neutrino scattering and electron-positron annihilation have emerged as significant new methods for the study of hadrons. Simple and elegant explanations of the phenomena observed in these new regimes have come from the quark model. Recently hadrons containing a new, heavier quark—the c-quark (for 'charmed')—have been discovered. The mass of the c-quark is thought to be roughly 1,500 MeV. The old quarks are much lighter: the u- and d-quarks may even be massless while the s-quark's mass is about 300 MeV. Because it is heavier the motions of the c-quark are easier to sort out. Quark model enthusiasts have been having a field day predicting (with great success) the properties of new 'charmed' hadrons composed of c-quarks together with the old u, d and s-quarks¹⁴.

The picture of relatively weakly interacting but permanently confined quarks permeates particle physics today. Unfortunately application of the picture to different problems rest on different *ad hoc* assumptions whose relationship to one another is often unclear. It is not a unified picture because we lack a unified theoretical description of confinement. At best, one would like to have a derivation of confinement from some deeper theoretical notions. At least one could hope to have an operational description of confined quarks consistent with the basic requirements of relativity and quantum mechanics. Recently there has been progress in both directions. Many of the ingredients of a fundamental theory of quark confinement perhaps have been discovered. These will be reviewed in the next section. The fundamental work unfortunately remains incomplete because the candidate theory cannot be solved: confinement cannot be demonstrated and the properties of hadrons cannot be calculated.

The theory

We have come very far in understanding the origins of the bizarre behaviour of quarks. The first step towards a solution was the realisation that hadrons possess a secret—a quantum

number which is essential in determining quark interactions but which never manifests itself openly in nature. Suppose in the days of atomic physics it had proved impossible to ionise neutral atoms, so only electrically neutral atoms were known. Eventually someone would have guessed that the constituents of atoms carry a hidden quantum number (this should not be confused with a 'hidden variable' which arose in a famous unsuccessful attempt to explain in purely classical terms the indeterminacy usually attributed to quantum mechanical effects)—electrical charge—which dictates the way they are put together in atoms but is never openly manifested in nature. In the case of quarks this hidden quantum number is called colour. It has nothing to do with ordinary visual colour, particle physicists just seem to like familiar names for their rather abstract notions. There are three possible quark colours which we may take to be 'red', 'green' and 'blue'. Each flavour of quark (u, d, s or c) comes in each of the three colours, but hadrons are always combinations of quarks with no net colour. They are colour neutral. (The reader may well wonder how fundamental quarks are now that we have twelve of them. What one calls a new particle is perhaps a matter of semantics. A particle with spin J has $(2J+1)$ different spin orientations which are not considered distinct objects. We likewise may not wish to call each different colour orientation of each flavour of quark a different particle¹⁵. In any case quarks will be more fundamental than hadrons if their interactions admit a simple, point-like description.)

Colour was originally introduced by Han and Nambu¹⁶ (another early version of the idea is due to Greenberg¹⁷) to resolve the symmetry mystery mentioned earlier: quarks in baryons seemed to occur only in symmetric configurations although Pauli had shown spin-1/2 particles must occur in antisymmetric arrangements. The solution was simple: the baryons are indeed symmetric in all the observed labels: spin, flavour and spatial configuration, but each quark carries an additional label—colour—and the baryons are antisymmetric arrangements in the colour label. So much for the symmetry mystery. Introducing a new quantum number is risky: the number of possible arrangements of quarks is greatly increased. Should all of these be observed in nature? In fact only a small selection of all conceivable arrangements of colour, flavour, spin and spatial quantum states have been found: all mesons may be classified as a quark and antiquark (Q $\bar{Q}$) of the same colour; all baryons may be classified as three quarks (Q³) made asymmetrical in colour. Remarkably these are the only combinations of Q $\bar{Q}$ or Q³ which are colourless. (Certain combinations of coloured quarks have no net colour just as certain combinations of charged electrons and protons have no net charge.) Colour could be introduced into the theory without predicting a vast, unwanted increase in the number of baryons and mesons if accompanied by a rule: all hadrons must be colour neutral.

There is an intimate relation between colour and confinement. A single quark, along with its fractional charge and baryon number, necessarily carries colour. So does any arrangement of two quarks, all of which also have fractional charge. However, a quark and an antiquark, which according to Table 1 have ordinary (integer) charges, also turn out to be the smallest arrangement of quarks with no net colour. The same goes for baryons—groups of three quarks have integer charge and baryon number and, remarkably, may be grouped into colourless configurations. In fact it can easily be shown that any combination of quarks and antiquarks which is colourless also has ordinary quantum numbers. Having learned this we can restate the confinement problem rather elegantly: quarks carry a new quantum number, colour, but for some reason all hadrons are colourless. Quark confinement has been reduced to colour confinement. Quarks must have interactions which select out colourless configurations. The case for colour as it stood in 1973 was summarised rather persuasively by Fritzsche, Gell-Mann and Leutwyler¹⁸, who also proposed a candidate for the actual quark-quark interaction (though the interaction itself seems to have been discovered independently by several workers^{19,20}.)

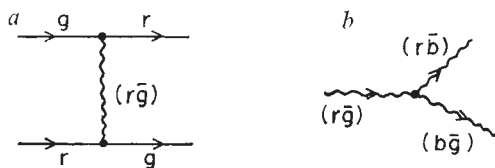


Fig. 5 A sample of interactions in quantum chromodynamics: *a*, red and green quarks scatter by exchanging a quantum of red-greenness; *b*, a red-green gluon emits a red-blue gluon and changes thereby into a blue-green gluon.

The theory of the interactions of electrons and photons, known as quantum electrodynamics (QED), is the most precisely verified theory in science. It is the model for construction of a theory of interactions consistent with both relativity and quantum mechanics. For some years there had been interest in generalisations of quantum electrodynamics known as Yang-Mills²¹ theories. QED is based on one kind of charge (electric) interacting with one kind of massless vector field (the electromagnetic field, whose quantum is the photon). In 1954 Yang and Mills formulated a theory of several charges interacting with many vector fields. The novel ingredient was that the vector fields carry the charges (in contrast to QED since the photon is neutral). Thus a particle could change its charge by emitting a field quantum which carries the compensating charge. The theory requires the quanta of the many vector fields to be massless. As in QED there are both electric and magnetic forces between charges. The Yang-Mills theory was not applied to strong interactions during the 1950s and 1960s because there are no massless strongly interacting particles. The possibility of confinement changes all this: the forces between quarks might be of the Yang-Mills type so long as the massless vector quanta (gluons) along with the quarks are permanently confined.

To return to the work of Fritzsche, Gell-Mann and Leutwyler, they supposed that the forces between quarks were of the Yang-Mills type described above. The colour quantum number plays the part of the charge. Quarks interact through colour electric and magnetic fields. With three quark colours it is necessary to have eight massless vector fields—'coloured gluons'. Eight gluons are required in order to maintain conservation of the colour quantum numbers and for all colours to be on an equal footing. A red quark may change into a green quark by emitting a quantum of red-greenness, that is, a red-green gluon. There are nine combinations of colours like red-green, but one combination is actually colourless and does not enter the interaction scheme. Figure 5 shows a sample of the basic interactions of the theory. Because the gluons themselves are coloured they interact with one another much like quarks. This theory turns out to be the only natural generalisation of quantum electrodynamics to a non-trivial group of conserved charges. For this reason it has become known as 'quantum chromodynamics' or QCD.

The case for QCD became compelling later in 1973 when D. Gross and F. Wilczek¹⁹ and H. D. Politzer²⁰ independently discovered a deep connection between QCD and Bjorken scaling. Generally forces become stronger at short distances. Everyone knows Coulomb's law: $F = e_1 e_2 / r^2$ provides a force which increases as charges are brought together. In fact the forces of electromagnetism are even stronger at short distances than Coulomb's law would indicate. At very short distances where relativistic and quantal effects are essential the effective charge, e , actually changes. It has long been known that in QED the effective charge increases at short distances. The MIT-SLAC electron scattering experiments however showed that quark-quark interactions become negligible at short distances within the proton. Gross and Wilczek and Politzer found that the gluon-mediated interactions in QCD, do grow weaker at short distances and vanish asymptotically at zero separation as required. No other realistic theory has this property of 'asymptotic freedom'. QCD is singled out as a candidate theory of the strong interactions.

The Yang-Mills theory of coloured quarks and gluons con-

tains all of the ingredients believed to be required of a fundamental theory of the strong interactions. Furthermore it is very precisely formulated. Nevertheless it has not been possible to address the basic question: does this theory lead to the confinement of quarks? The reason for this predicament lies in the deep distinction between formulating a theory of interacting fields and applying it to most physically interesting problems. Theories are formulated 'locally', that is, the nature of the interaction between fields, here quarks and gluons, is specified at an arbitrary point in space and time. Hadrons are, however, extended regions of space in which quark and gluon fields interact with one another constantly. The comparatively simple local interactions can give rise to an arbitrarily complex global structure for a hadron.

Some approximation must be made. Quantum electrodynamics is the only example we have of a theory which is consistent with both relativity and quantum mechanics and for which approximate solutions can be found. The key to approximation is the weakness of the electromagnetic force. The dimensionless measure of its strength is the 'fine-structure constant' $\alpha = e^2 / \hbar c$ (c is the speed of light, e is the electronic charge and $\hbar$ is Planck's constant). Since α is about $1/137$, things may be calculated perturbatively. To a first approximation there are only free electrons and photons. To next order two electrons have a small probability amplitude ($\sim 1/137$) to interact by the exchange of a photon, and so on. The forces between particles generated in this fashion characteristically fall off with increasing distance. Systems of interacting particles can be assembled by bringing the components together from large distances where their interactions are negligible.

This won't work for a theory of quarks. They are supposed to be permanently confined. There is no sensible limit in which the quarks can be treated as isolated particles. Over very short distances inside a proton quarks interact quite weakly because of 'asymptotic freedom' and a form of perturbation theory can be used to calculate their behaviour. But only a few properties of hadrons are sensitive exclusively to what happens at short distances. Most involve distances of the order of the proton's radius where interactions must begin to become strong if quarks are to be confined. The usual perturbative treatment begins with free quarks and gluons which, if the programme is successful, must miraculously disappear in the finale.

A new approximation scheme is needed. Efforts in this direction have yet to be successful but the attempts themselves have introduced many exciting new ideas into theoretical physics. One approach is to alter the structure of space-time in order to make calculations more convenient. In this work, pioneered by K. Wilson²² and by J. Kogut and L. Susskind²³, space or both space and time are replaced by a lattice of discrete points. To lowest order quarks sit on a single lattice site. To higher orders quarks move from site to site: the hadron spreads out. It is not difficult to show that in this approach to QCD quarks can never be separated from one another. Of course, space-time is not a lattice. There is evidence for its continuity down to distances of the order of $1/100$ the proton's radius²⁴. The burden on the lattice approach is to determine whether its predictions are consequences of the original quark gluon theory or artefacts of approximating space-time as a lattice. This dilemma remains unresolved today.

There has been much excitement recently concerning non-perturbative solutions to field equations. Often classical theories of interacting fields admit exact solutions where the field configurations are in no sense small perturbations about the trivial solution—empty space. According to their properties such solutions are known as 'solitons' or 'instantons'. Theories which admit these solutions seem to be qualitatively different from those which do not. Ordinary quantum electrodynamics seems not to. Quantum chromodynamics does, and it is thought that the existence of these solutions is associated with confinement.

An association like this occurs in the theoretical description of magnetic fields in superconductors. When metal in a magnetic field is cooled and becomes superconducting the lines of

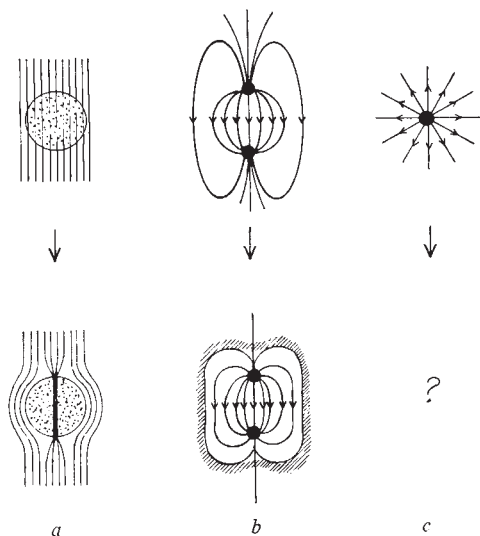


Fig. 6 A superconductor analogy for confinement. *a*, When an ordinary metal in a uniform magnetic field is cooled and becomes a superconductor the magnetic flux lines are excluded from the superconducting region though some may be trapped in 'flux tubes' (Meissner Effect); *b*, quark confinement may be due to a colour-electric Meissner Effect in which the vacuum excludes colour electric flux. A quark-antiquark system is confined to a 'bag' but a single quark cannot exist since its flux lines have no place to end and cannot stretch out to infinite distance.

magnetic flux are expelled from the interior of the superconductor, though some may be trapped within tubes of 'normal' metal within it. This effect, illustrated in Fig. 6*a*, is known as the Meissner effect²⁵. The magnetic field lines are, after a fashion, confined. Although this strange behaviour is caused by the interactions of the electrons within the metal, it cannot be obtained by treating them perturbatively. In an ordinary metal electrons move about rather freely much like a gas in a box. In superconductors there is a configuration of interacting electrons which is radically different and lower in energy than the free 'gas' configuration²⁶. Since it is lowest in energy it is the preferred (ground) state of the system. All of the bizarre properties of superconductors are attributable to this non-perturbative ground state.

Similar non-perturbative solutions to Yang-Mills theories were found by Polyakov and his collaborators²⁷. These and other recently discovered non-perturbative solutions to Yang-Mills theories have excited intense interest. As yet no one has succeeded in establishing a connection between them and the confinement problem. The Meissner effect seems much simpler than QCD.

The analogy with superconductivity can be pushed further. S. Mandelstam has particularly emphasised this point of view²⁸. A superconductor expels magnetic flux lines. Suppose empty space (the vacuum) expels colour-electric flux lines. There is a Gauss's law in QCD as in electromagnetism. It requires that lines of colour-electric flux, which begin and end on coloured quarks and gluons, cannot be cut. The colour charge within a given region of space can be measured by surrounding the region with an imaginary surface and counting the number of colour flux lines crossing the surface. If, for some unknown reason, the vacuum expels colour flux lines then all coloured objects and all their attendant flux lines must be confined to some 'normal' region. An isolated quark could not exist since it is the source of flux lines which would have to stretch out for infinite distances. Only configurations with no net colour could exist since they are not required to have flux lines extending to infinity. If this could be shown then QCD would give us an explanation for quark confinement. The 'normal' and 'colour-electric superconducting' states are illustrated in Fig. 6*b* and *c* for a quark-antiquark pair and for a single quark. Perhaps the non-per-

turbative solutions of QCD will have the effect of making the vacuum behave towards colour-electric flux the same way that a superconductor behaves towards magnetic flux. As present this is merely speculation.

The model

Long before the modern explanation of superconductivity and the Meissner effect were known, Ginsberg and Landau made a model to describe superconductivity without attempting to derive it²⁹. Since confinement seems to be at least as hard a problem, my collaborators and I at MIT have taken a similar approach to it³⁰. It turns out to be easy to write down a simple and elegant model incorporating confinement. Our approach is more radical than that of conventional quantum field theory. Rather than starting with quarks and gluons everywhere, and then trying to confine them, we start by assigning coloured fields only to the inside of hadrons—since that is where they seem to be. This makes it necessary to distinguish the space inside a particle from that outside it—the hadron must be an 'extended', geometrical object. The shape of a hadron cannot in general be fixed. Rigid objects are not consistent with relativity. If an object is hit at a point on its surface it is not possible for the object to recoil rigidly because other points on the surface would have to have learned of the impact instantaneously. Relativity requires that no information travel faster than the speed of light. Each point on the boundary must be free to move. The equations which describe the motion of the boundary of an extended object must be local in order not to violate relativity.

It is possible to distinguish an otherwise empty region of space in a way consistent with relativity by subjecting the boundary of the region to a constant pressure, B where B may be thought of as a pressure exerted by otherwise empty space on the hadron. There is a simple, classical picture of the dynamics of such an object. A hadron is like a bubble of gas in a uniform, isotropic perfect fluid. The dynamics of a gas bubble is determined by the balance between the external pressure of the fluid and the thermodynamic pressure of the confined gas. A hadron is such a bubble containing only a few quanta instead of the vast number of particles in a macroscopic bubble. Quanta confined to a region of size Δx have—by Heisenberg's uncertainty principle—a momentum $\Delta p \sim \hbar/\Delta x$ and therefore exert a counter-pressure. This quantum pressure keeps the hadronic region open by pushing against the external pressure B . The pressure B fixes the size of hadrons. For protons to be of radius 1 fm (1 fm = 10^{-13} cm), B is required to be about 60 MeV fm⁻³ (or 8×10^{28} atmospheres). This extended region of space is called a 'bag'. Since the model was developed at MIT it has become known as the MIT bag model.

Quarks and gluons are the quanta of coloured fields. What about a one quark bag which would be coloured and have fractional charge? States like this never appear in the model: by construction, colour electric fields, like quarks, exist only inside the bag; but by the analog of Gauss's law, colour electric flux lines must emanate from a bag with net colour. The only consistent solution is for all bags to have no net colour. In fact an infinite energy barrier prevents a colour neutral bag from fissioning into two coloured bags. The situation is illustrated in Fig. 7. Suppose in a colour singlet bag, the colours C and $-C$ are separated by a finite distance. The colour electric field lines which connect the charges C and $-C$ exist only in a finite volume V since (in this model) it costs an energy $BV > 0$ to create the space which carries them. Hence at a point between the charges, the fields span a finite cross section A and by Gauss's law, $C = EA$ where E is the mean colour field strength in the cross section. The energy stored in a colour electric field charges as half the square of the field strength, therefore the energy per unit length is $\frac{1}{2}E^2A = \frac{1}{2}C^2/A$. Separating the colours by a distance l costs an energy $\frac{1}{2}C^2l/A$. Fission requires $A \rightarrow 0$ and $l \rightarrow \infty$; it requires an infinite energy to separate colours. Thus colour-, and hence quark-confinement is automatic and comprehensible in this model.

The particular virtue of this approach is that it offers the hope of calculating hadron properties perturbatively³¹. Quarks are arranged into colourless states *ab initio*. The agent responsible for this—a universal pressure—is simple in principle. With such a simple starting point it has proved possible to reconcile some of the apparently unrelated applications of the quark model with one another. Studies of the model reveal two broad classes of hadron which may be called 'spherical' and 'deformed'³². Hadrons made of u, d and s quarks with small amounts of angular momentum form spherical bags. To lowest order such a hadron (the proton for example) is a bag of free quarks (as seen in the MIT-SLAC highly inelastic electron scattering experiments)³³. To next order, the gluon mediated interactions modify the masses and other properties of the hadrons³¹. If, on the other hand, the hadron contains heavy quarks like the newly discovered charmed quark or if it possesses large amounts of angular momentum, it will deform. Deformed hadrons are actually simpler to picture. One is shown in Fig. 7b. Consider a meson made of charmed quark and antiquark. Heavy quarks move about less and exert little pressure, but the bag does not collapse around the heavy quarks because they are sources of colour flux lines. The colour fields exert pressure perpendicular to the lines of electric flux. The bag therefore deforms about the flux lines connecting the quark and antiquark. Angular momentum has the same effect; centrifugal force throws quark and antiquark apart and leads to deformation. Light quarks with little or no angular momentum are smeared out throughout the bag. On the average there is no source for colour flux and therefore no axis for deformation.

Detailed calculations of hadron properties have been carried out with the model³¹⁻³⁴. One example will suffice to illustrate the sort of success which it has had³⁴. The masses of spherical hadrons are determined by the quark-gluon dynamics in terms of two fundamental parameters: B , which sets the scale; and a 'coupling constant', α_c which measures the strength of the quark-gluon interaction. The same parameters determine the masses of deformed hadrons although the basic dynamics is quite different. In deformed hadrons the glue is present in lowest order and balances the pressure B , while in spherical hadrons only quarks are present in lowest order and gluon interactions cause small modifications in higher order.

Deformed hadrons have long been known to form families with different angular momentum and mass but otherwise similar properties. In nuclear physics similar families are called rotational bands. In subnuclear physics they are called 'Regge trajectories'. Hadrons on the same Regge trajectory have a

Fig. 7 Hadron dynamics. *a*, A colour neutral spherical meson; *b*, energy is delivered to the meson causing the quark and antiquark to move apart; *c*, colour-electric flux lines connecting quark to antiquark prevent separation; *d*, eventually the energy density in the region between the quark and antiquark becomes sufficient to enable another $Q\bar{Q}$ pair to materialise. These provide new sources and sinks of colour electric field. The system shown in (*d*) will soon fission into two colourless mesons.

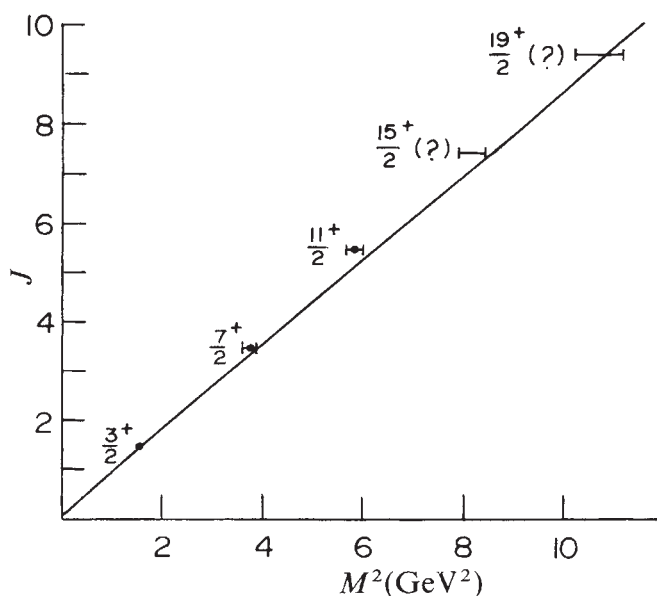
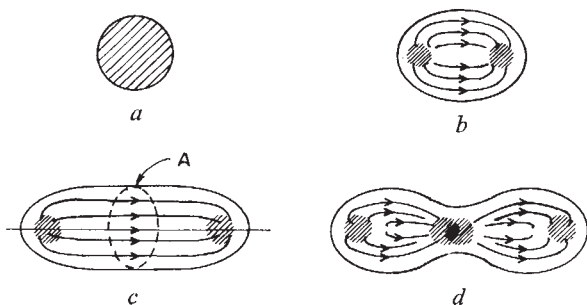


Fig. 8 The Δ -Regge trajectory: the points represent observed hadrons believed to be excitations of the Δ -baryon (see Fig. 1).

remarkably simple relation between mass and angular momentum: $J = \alpha' M^2 + \alpha_0$, where J is the total angular momentum of the hadron, M is its mass and α' and α_0 are called the Regge 'slope' and 'intercept' respectively. One of the best known Regge trajectories is shown in Fig. 8. Experimentally it is found that the Regge slope α' is the same for both baryons and mesons and equals 0.9 GeV^{-2} .

In the bag model hadrons with much angular momentum do obey the relation $J = \alpha' M^2 + \alpha_0$ and α' is the same for both baryons and mesons. The universality of α' is natural. The mass of a spinning hadron is determined by a dynamic balance of the gluon field pressure (involving α_c) and the universal pressure B . A spinning meson has opposite colours on either end. A spinning baryon has two quarks on one end and one on the other. The two quarks, however, have antiquark colour in order to combine with the remaining quark to make a colourless hadron. Thus the colour fields in a spinning meson and baryon are the same making α' universal. α' is found to be a simple function of α_c and B . To lowest order: $1/\alpha' = \pi^{3/2} \sqrt{8\alpha_c B/3}$. Remarkably, if α_c and B are adjusted to fit the masses of light, spherical hadrons, then α' comes out 0.88 GeV^{-2} . Two heretofore disjoint aspects of hadron phenomenology are therefore only different reflections of the same underlying physics.

At present the bag model has some important limitations. For example, it is not known whether it can be made consistent with quantum mechanics. All the applications discussed in the literature have made use of a semi-classical approach similar in spirit to Bohr's treatment of the hydrogen atom. In general, relativistic field theories are known to be consistent with quantum mechanics only when studied order by order in ordinary perturbation theory. But as we have noted, perturbation theory has little or nothing to say about confinement. The bag model abandons conventional perturbation theory in search of a starting point closely related to the observed phenomena of confinement. The price seems to be the loss of much of the useful conceptual machinery of quantum mechanics.

Subnuclear physics is in a rather peculiar state at present. There is wide agreement as to the ingredients in a prospective theory of hadrons: quarks, colour, gluons and the Yang-Mills interaction, to name the most obvious. Yet there are no

precise, unequivocal and successful calculations of anything to bolster our faith in the theory. In QCD it is not even clear where to begin. If confinement is put in by hand by introducing the bag pressure B then many approximate calculations are possible. The resulting model is especially successful at understanding qualitative features of hadron behaviour. But even having abandoned the attempt to derive confinement one is still left with an extremely complex dynamical system which defies rigorous treatment and quantisation. It is not surprising that our description of hadrons remains in large measure incomplete. Nothing like the permanent confinement of quarks has been encountered before in the study of the structure of matter. Old approximation schemes fail, new ones develop slowly.

What is surprising is the emergence of a candidate theory of hadrons so soon after the realisation that quarks may be permanently confined to the interior of hadrons. It is particularly remarkable since none of the basic ingredients in the theory has ever been observed directly. Instead their existence has been inferred by brilliant and subtle qualitative analyses. The discovery of colour by way of the quark symmetry puzzle and the necessity of gluons and Yang-Mills interactions to understand the paradoxical 'free yet confined' behaviour of quarks are cases in point. The theory which has emerged is very precisely formulated and tightly constrained despite the rather indirect way its ingredients have been established. It offers the hope of explaining hadron properties in precise detail although at present only approximate calculations are possible. It is also very pretty, at least to particle physicists.

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articles

The location of the polio genome protein in viral RNAs and its implication for RNA synthesis

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Evidence is presented that a small protein (VPg) is covalently attached to the 5'-terminal oligonucleotide VPg-pU-U-A-A-A-C-A-Gp of the polio genome, to nascent strands of the polio replicative intermediate and to poly(U) of minus strands. A model of polio RNA replication is proposed implicating VPg in initiation of RNA synthesis, possibly as primer.

THE 5'-terminal structure of a ribonucleic acid may reveal mechanisms involved in the biosynthesis of that RNA. For example, the 5' end of coliphage RNA is a nucleoside triphosphate indicating that initiation of phage RNA synthesis occurs *de novo*¹. The genomes of plus strand RNA viruses of eukaryotic cells, on the other hand, are 'capped' at the 5' end with m⁷G(5')ppp(5')Np . . . which is a post-transcriptional modification². To date, two exceptions to this group are known: genome RNA of satellite tobacco necrosis virus which terminates in (p)ppA-G-U (ref. 3), and genome RNA of picornaviruses which contain neither a terminal triphosphate nor a capping

group. In fact, previous efforts to identify any 5' end of picornavirus RNA were unsuccessful⁴⁻⁹. Recently, we have identified a low molecular weight protein (called VPg) covalently linked to polio virion RNA and have suggested that this protein may be attached to the 5' end of the genome^{10,11}. In this communication we present experimental evidence for the association of the protein with the 5' end of virion RNA. We also show that VPg is bound to nascent strands of the polio replicative intermediate and to the 5'-terminal poly(U) of polio minus strands. This novel structural feature of poliovirus RNAs permits us to suggest a model of replication which implicates the protein in the initiation of RNA synthesis.

Genome-linked protein VPg at the 5' end of virion RNA

The location of VPg within the viral genome can be determined by comparing the number of phosphates remaining on the nucleotidyl-protein obtained after digestion of the RNA with the enzymes RNase T2 or nuclease P1. As previously shown, digestion of purified virion ³²P-RNA with RNase T2 yields mononucleotides and a small protein of approximately 6,000

molecular weight (called T2-VPg), which is linked to pUp (ref. 11). As illustrated in Fig. 1a, this nucleotidyl-protein, [(aa)_n-ZpUp, where Z is the hypothetical linkage group between the protein and nucleotide], will contain two phosphate groups independent of its location on the RNA. Nuclease P1 is an endonuclease which yields nucleoside 5'-phosphates¹². When virion RNA is cleaved at low enzyme concentrations (0.4 mg ml⁻¹, 2 h at 37 °C), the linkage between Z and pU is stable. As illustrated in Fig. 1a the nucleotidyl-protein produced by nuclease P1 (P1-VPg) would be predicted to contain only one phosphate if it were terminally bound and at least two phosphates if it were internally bound.

The isolation of P1-VPg can be seen in Fig. 1b which shows that the nucleotidyl-protein appears in the flow-through fractions of the ion-exchange column. T2-VPg appears at the same position in this column¹¹. P1-VPg and T2-VPg also comigrate by electrophoresis on polyacrylamide gels (for conditions, see ref. 11). Analysis of the number of phosphate groups on P1-VPg required proteolysis due to the affinity of VPg to surfaces¹⁰. Accordingly, we digested ³²P-P1-VPg with Pronase which yielded a single phosphorylated product ³²P-P1-Y, with a net charge of -1 at pH 5 (Fig. 1c). P1-Y is resistant to alkaline phosphatase (data not shown) indicating the absence of a phosphomonoester. Isolation of the proteolytic product of ³²P-T2-VPg is shown in Fig. 1d. ³²P-T2-VPg has a net charge of -2 at pH 5 (ref. 11). Half of its label is sensitive to alkaline phosphatase (data not shown). Snake venom 3'-exonuclease yields pUp from T2-Y and pU from P1-Y (Fig. 2a, b). The data are compatible with ZpU being the structure of P1-Y. We therefore conclude that P1-VPg has the structure (aa)_n-ZpU and originated from the 5' end of the viral RNA (Fig. 1a). The covalent attachment of a protein to the 5' end of genome RNA represents a novel feature in the molecular biology of viruses.

Prolonged digestion of ³²P-T2-VPg with Pronase occasionally yielded T2-Y and a by product (T2-Y') in low yield, which migrates faster than T2-Y during electrophoresis on Whatman 3MM paper. Incubation with aminopeptidase converts T2-Y quantitatively to T2-Y' (Fig. 3). T2-Y' yields pUp with snake venom exonuclease (not shown) as does T2-Y and is therefore a derivative of T2-Y from which presumably an amino acid or another ligand has been removed. The structure of T2-Y' (Z'pUp), which harbours the linkage between VPg and poliovirus genome, is currently under investigation.

Isolation of a 5'-terminal, protein-linked oligonucleotide

To isolate a 5'-terminal fragment produced by RNase T1, we took advantage of the fact that VPg is positively charged as it migrates toward the cathode during paper electrophoresis at pH 3.5 or pH 7.5 (ref. 10). Thus, the protein-linked fragment would be the only oligonucleotide in an RNase T1 digest whose net charge would be changed by Pronase treatment permitting a 'shift out' experiment originally proposed by Tomlinson and Tener¹³. First, we separated an RNase T1 digest of virion [amino acyl-³H]RNA by ion-exchange chromatography in 7 M urea (data not shown). Tritium counts were found only in a peak containing trinucleotides, an observation suggesting that the 5'-terminal oligonucleotidyl-protein has a net charge of -4. This was confirmed by repeating the experiment with virion ³²P-RNA (Fig. 4a). Material in peak -4 was desalted and digested with RNase T2 which yielded T2-VPg (Fig. 4b). The nature of this T2-VPg was verified by Pronase and nuclease digestion as described in Figs 1d and 2a. When the material in peak -4 (Fig. 4a) was digested with Pronase, a new oligonucleotide with increased negative charge was produced whereas the trinucleotides remained unchanged (Fig. 4c). The shoulder of the peak (≥ -10) in Fig. 4c indicated to us heterogeneity of the oligonucleotide, the extent of which changed from experiment to experiment. The material in peak (≥ -10) was pooled and separated into two spots ('oligo I' and 'oligo II') by two-dimensional gel electrophoresis (data not shown;

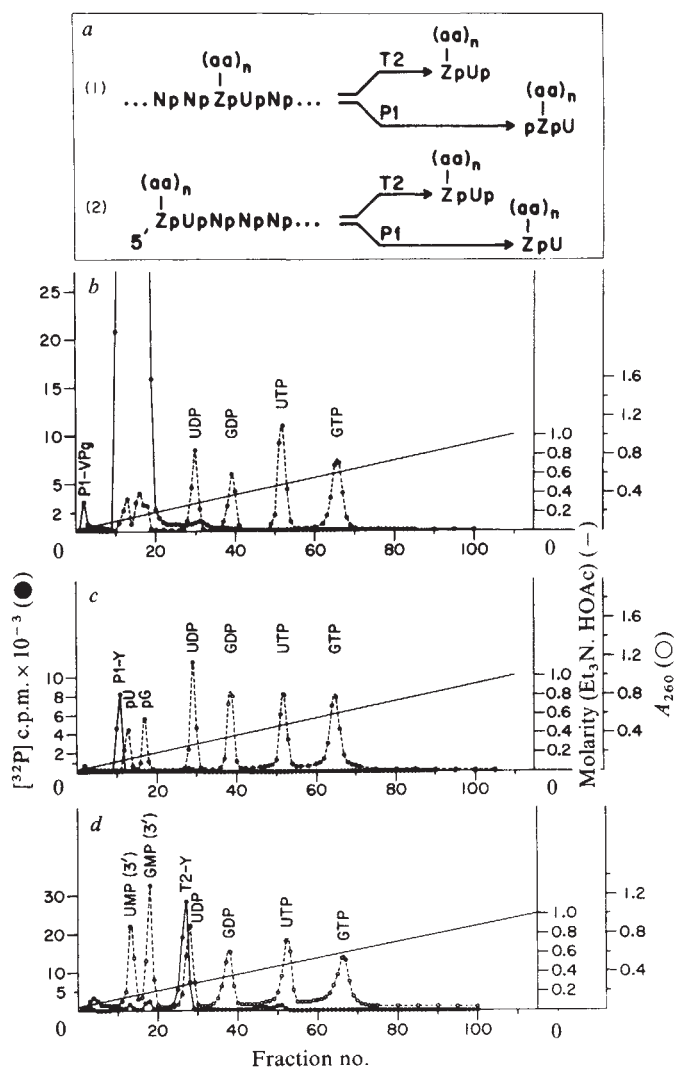


Fig. 1a, Hypothetical cleavage scheme for poliovirus RNA. The genome-linked protein may be bound to the RNA (1) internally or (2) terminally through a linkage group Z which has not yet been identified¹⁴. If internal, Z would be a nucleoside. If terminal, Z could be a nucleoside, one or more amino acids, or any other linkage group. Digestion of the RNA with RNase T2 or nuclease P1 would yield different nucleotidyl-proteins (T2-VPg or P1-VPg) which can be identified through the difference of their phosphate esters. **b-d,** Separation of digestion products of poliovirus RNA by column chromatography at pH 5 in the presence of marker nucleotides. Details as described in ref. 5. **b,** 8×10^7 c.p.m. ³²P-RNA were digested with 20 µg nuclease P1 (Yamasa) in 50 µl of 0.05 M ammonium acetate, pH 6, for 2 h at 37 °C. 8×10^8 c.p.m. nucleotidyl-protein, P1-VPg, were recovered by lyophilisation which is 52% of the theoretical yield (yield was corrected as explained in Table 1). In other experiments the yield was as high as 75%. **c,** ³²P-P1-VPg was digested in 200 µl of 0.05 M Tris-HCl, 5 mM sodium phosphate, pH 8, 0.1% sodium dodecylsulphate, with 10 µg Pronase (Sigma Protease type VI) for 20 h at 37 °C. **d,** Digestion of ³²P-T2-VPg with pronase as in Fig. 1c. P1-Y and T2-Y were recovered by lyophilisation.

see ref. 14). 'Oligo I' (which we found to elute ahead of oligo II from the ion exchange column; Fig. 4c) and 'oligo II' were recovered from the gel and characterised by further enzymatic analysis (see legend to Fig. 5). Separation of digestion products (Fig. 5) revealed that the oligonucleotides differ only in their amino acyl component resulting from overdigestion with Pronase: the nucleotides of oligo I are linked to Y, those of oligo II are linked to Y'. On the other hand, we repeatedly observed that the amounts of label in the digestion products of the 5'-terminal oligonucleotide disagreed with the expected yields (Table 1). For example, the radioactivity found in Y was

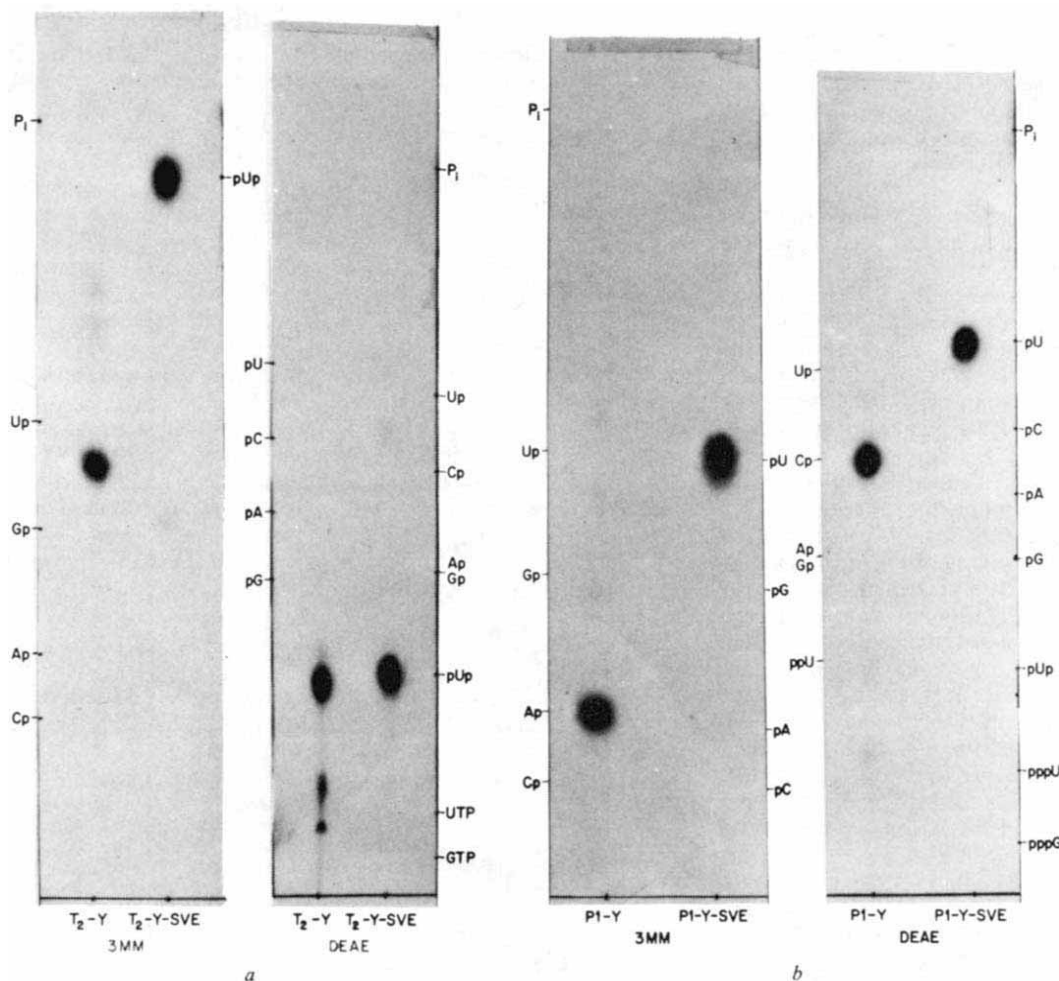
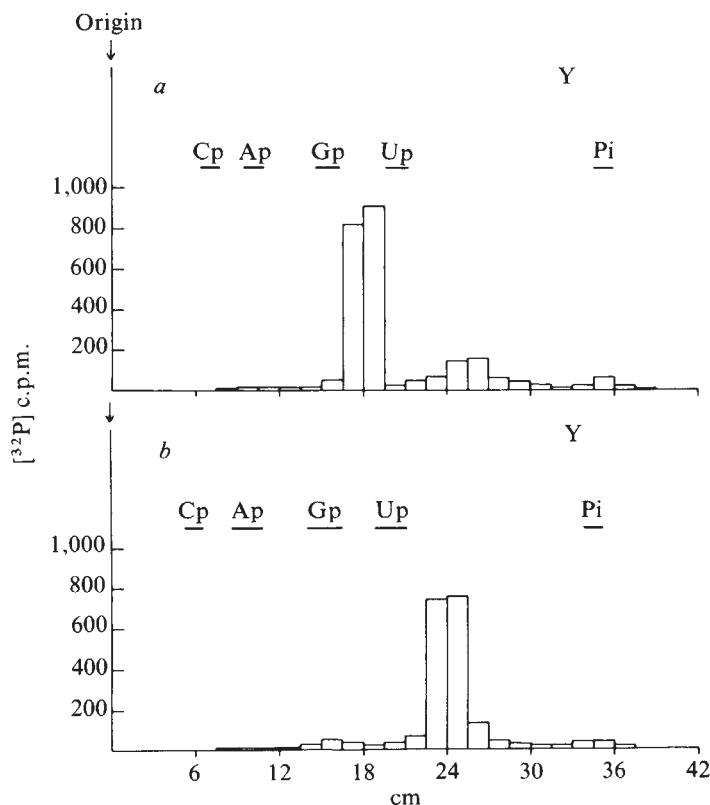


Fig. 2 Paper electrophoresis of ^{32}P -T2-Y and ^{32}P -P1-Y (see Fig. 2d, c) and their digestion products with snake venom 3'-exonuclease (SVE). a, T2-Y and b, P1-Y were treated with 0.5 μg purified exonuclease (gift from Dr M. Laskowski, Sr.) in 10 μl 0.02 M Tris-HCl, pH 7.5, 0.005 M MgCl_2 for 1 h at 37° . Electrophoresis was carried out in 5% acetic acid, adjusted to pH 3.5 with pyridine, on Whatman 3MM paper for 1.5 h at 4000 V and on DEAE-paper for 2.5 h at 3000 V. The labelled compounds were visualised by autoradiography. The identity of pUp in a and pU in b was verified by two-dimensional thin-layer chromatography.

almost twice as high as that found in ApGp after RNase A digestion of oligo II, although both products contain two phosphates (Table 1). The discrepancy can be explained in that the polio ^{32}P -RNA used in our study was not uniformly labelled. This was detected as follows. The base composition of the virion ^{32}P -RNA, when analysed by the isotope dilution method after complete digestion with RNase T2, is Cp = 24.3%, Ap = 30.1%, Gp = 22.5%, and Up = 23.1%, reflecting the base composition of unlabelled RNA¹⁶. A different base composition, however, is obtained after digestion of the ^{32}P -RNA with nuclease P1 or snake venom exonuclease which yield 5'-monophosphates (pC = 21.2%; pA = 30.5%; pG = 12.7%; pU = 35.6%). These observations suggest that the precursor pools for viral RNA synthesis were not fully equilibrated during growth of the virus. Instead, the specific radioactivity of ^{32}P -GTP and ^{32}P -CTP in the infected cell was lower than that of ^{32}P -UTP. As outlined in Table 1, we have corrected the observed radioactivity of oligonucleotides derived from the 5' end of polio ^{32}P -RNA for the difference of the specific radioactivity of their individual monophosphates. The corrected values are close to those expected. Based on the cleavage products found and because Y and Y' contain pUp, we conclude that the 5' end of the poliovirus genome is homogeneous and has the sequence VPg-pU-U-A-A-A-A-C-A-Gp.

Fig. 3 2.5×10^3 c.p.m. each of ^{32}P -T2-Y (see Fig. 1d) were incubated for 2 h at 37°C , a, in 10 μl 0.05 M sodium phosphate, pH 8, or b, in the same buffer with 0.5 units aminopeptidase M (Sigma). Samples were electrophoresed on Whatman 3MM paper as described in Fig. 2. 1.5 cm strips of the paper were monitored for radioactivity.



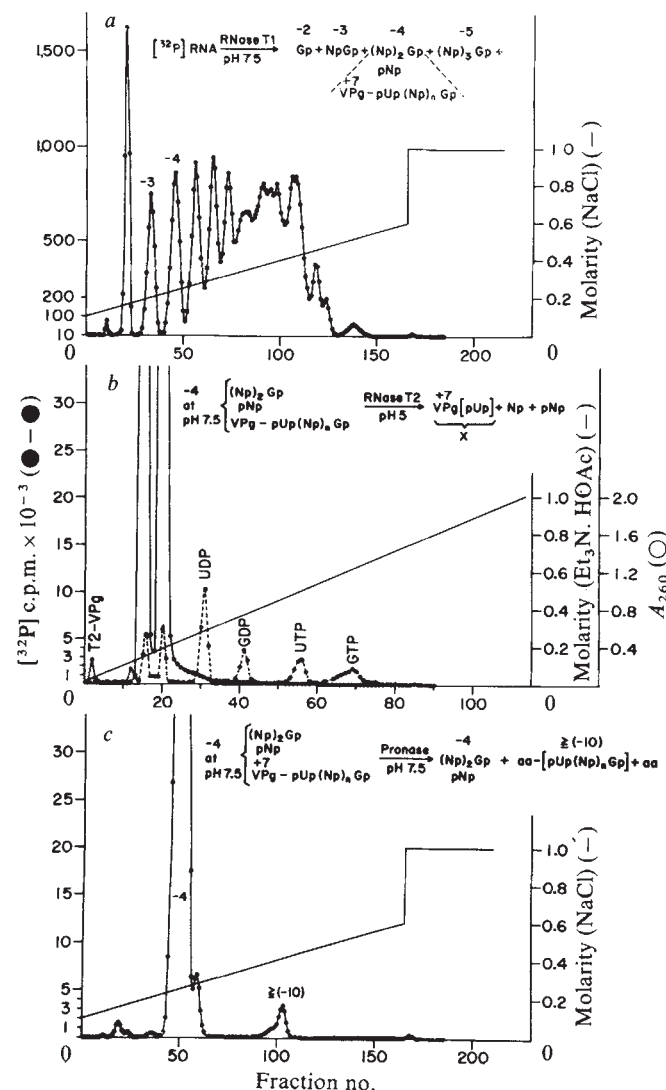


Fig. 4 Isolation of the 5'-terminal oligonucleotide of poliovirus RNA. *a*, 400 µg (4×10^8 c.p.m.) of poliovirus ^{32}P -RNA were digested with 50 units of RNase T1 (Calbiochem) in 200 µl 0.05 M Tris-HCl, pH 7.5, 0.002 M EDTA for 2 h at 37 °C. The mixture was applied to a DEAE-Sephadex A25 column and the components eluted with a linear gradient (200 ml) of 0.1–0.6 M NaCl in 0.02 M Tris-HCl, pH 7.5, 7 M urea. *b*, Peak -4 of Fig. 4*a* was desalted, digested with RNase T2 and chromatographed on a DEAE-cellulose column in triethylammonium acetate, pH 5, as described in Fig. 1*b*. X corresponds to the nucleotidyl-protein T2-VPg. *c*, Peak -4 of Fig. 4*a* was desalted, digested in 50 µl buffer with Pronase as described in Fig. 1*c*, and chromatographed on DEAE-Sephadex as described in Fig. 4*a*. Peak (≥ -10) was pooled and separated into two oligonucleotides ('oligo I' and 'oligo II') by two-dimensional gel electrophoresis (see text).

From the net charge of phosphates of the 5'-terminal oligonucleotidyl-protein (calculated to be -11) and its elution at peak -4 (Fig. 4*a*) we estimate that VPg must have a net positive charge of at least +7 at pH 7.5. The positive charge and the affinity to surfaces of polio VPg resemble properties of small nuclear proteins, for example histones of the host-cell. Available evidence suggests, however, that VPg is virus coded as it can be labelled with amino acids 3 h after infection when host protein synthesis is inhibited¹¹. Moreover, we have identified a protein linked to genome RNA of another picornavirus, the encephalomyocarditis virus, which was grown in HeLa cells as was poliovirus. The polio-VPg and the EMC-VPg, although isolated from the same host cell, are slightly different in their molecular weights, an observation supporting our notion that the VPg's are virus specific proteins (Golini, A. N. and E. W. unpublished).

VPg is linked to nascent strands of polio RI and to minus strands

The polio replicative intermediate (RI) has been identified as the only structure involved in the synthesis of viral single stranded progeny RNA (see ref. 17 for review). We have, therefore, analysed RI for VPg since the presence of VPg might identify the step in viral replication during which the protein is attached to the viral RNA. ^{32}P -RI was isolated from polio virus-infected HeLa cells as previously described¹⁸. Absence of free plus strand RNA was shown by gel electrophoresis in 1% Agarose (data not shown; compare ref. 18). Digestion of denatured ^{32}P -RI with RNase T2 and separation of the products by column chromatography as in Fig. 1*b* revealed that the structure does not contain pppNp, ppNp or capping groups (data not shown). Some material eluting at the position of nucleoside diphosphates was analysed by paper electrophoresis and found to contain all 4 pNp's: we assume this material originated from suprious ends in the RI. Material eluting in the void volume was shown to be T2-VPg by (1) subsequent gel electrophoresis, in which it co-migrated with virion RNA-derived T2-VPg, and (2) by digestion with Pronase and snake venom exonuclease which yielded T2-Y and pUp, respectively (data not shown). The number of nascent

Fig. 5 Separation of digestion products by electrophoresis on DEAE-paper in pyridinium acetate, pH 3.5 (see Fig. 2) at 3,000 V for 4 h. Oligo I (*a*) and Oligo II (*b*) (see Fig. 4*c*), which had been purified by two-dimensional gel electrophoresis, were digested in 10 µl 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA with RNase A for 1 h at 37 °C, or in 10 µl 0.05 M ammonium acetate, pH 4.5, 1 mM EDTA with 0.1 unit RNase T2 for 30 min at 37 °C. Oligo II (*b*) was digested in 10 µl acetate buffer, pH 4.5, with 0.05 units RNase U2 for 1 h at 37 °C. Radiolabelled compounds were visualised by autoradiography. The identity of oligonucleotides was verified by secondary digestions with RNase T2. Note that digestion with RNase U2 is incomplete in these conditions and yields 2',3'-cyclic phosphates¹⁵. For quantitation of the products see Table 1.

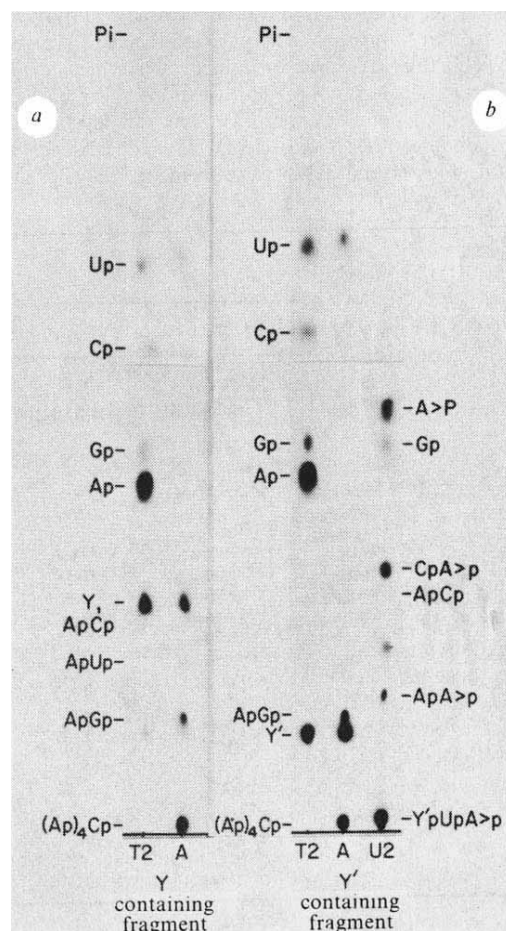


Table 1 Products of nuclease digestion of the polio 5'-terminal oligonucleotide

RNase T2*			RNase A*			RNase U2*†
Products	Yield (c.p.m.)	Corrected‡	Products	Yield (c.p.m.)	Corrected‡	Products
Y' (Z' pUp)	1,566	1,018	A ₄ Cp	1,990	2,029	Y'-UA > p
Ap	1,957	2,310	Y' (Z' pUp)	996	647	AA > p
Gp	422	485§	AGp	450	657§	Unknown
Cp	534	528	Up	438	434	CA > p
Up	699	692				Gp
						A > p

*Digestion of oligo II and identification of products as described in Fig. 5.

†Digestion with RNase U2 is incomplete. The unknown spot was absent in other experiments.

‡The observed counts for each phosphate are corrected for the deviation from uniform labelling of the RNA by multiplying the values with a factor f ($f = [\% \text{ Np in total RNA after RNase T2 digestion}] / [\% \text{ pN in total RNA after nuclease P1 digestion}]$). For values of base compositions see text. Factors of each 5'-phosphate are: pC = 1.15; pA = 0.99; pG = 1.77; pU = 0.65. For example, the value observed for Y' (Z' pUp) is corrected by multiplying it with 0.65 since both phosphates originate from pU.

§Assuming that the nearest 3'-neighbour is C. The values are slightly lower if A or U are the nearest neighbours.

strands in RI can be derived from the resistance of the structure to digestion with RNase A (ref. 17). Based on an RNase A resistance of ^{32}P -RI of 36.4%, we calculated that the structure must contain on the average seven nascent RNA strands or 5.5 full length equivalents (the template strand included). This corresponds to 42.3×10^3 nucleotides assuming 7.7×10^3 nucleotides per polio virion RNA molecule. 32×10^6 c.p.m. of ^{32}P -RI yielded 12,774 c.p.m. of ^{32}P -T2-VPg. If corrected for the higher than average specific radioactivity of the two UTP-derived phosphates in VPg, these counts correspond to five T2-VPg molecules which is 63% of the theoretically expected value of eight VPg's. Since losses are encountered during the isolation procedures of the adhesive protein¹⁰ we suggest that each nascent strand of polio RI is linked to VPg at the 5' end.

Evidence for VPg on minus strand RNA was obtained as follows: ^{32}P -RI (ref. 18) or ^{32}P -RF (refs 19, 20) were denatured and annealed to excess unlabelled virion RNA. This removed most labelled plus strand RNA (including the nascent strands) from minus strand RNA²⁰. The hybrid double strands were then purified by gel filtration and the 5'-terminal ^{32}P -poly(U) isolated by affinity chromatography after RNase T1 digestion²⁰. The poly(U) thus obtained contained in a typical experiment 1 VPg, 45 Up and 1-4 mol of Gp, Cp and Ap. Presence of bases other than Up would be expected²⁰. The low levels of Gp, Cp and Ap, on the other hand, clearly indicate absence of contaminating plus strand RNA. The yield of ^{32}P -VPg, however,

is higher than expected since the average length of poly(U) has been reported to be 60-90 nucleotides¹⁸⁻²⁰. We have, at present, no explanation for the high yield. ^{32}P -VPg was isolated from ^{32}P -poly(U) by column chromatography as described in Fig. 1b. It had the same mobility in polyacrylamide gels as virion RNA-derived VPg and yielded after digestion with Pronase and snake venom exonuclease T2-Y and pUp, respectively (data not shown). We conclude that polio minus RNA is linked at the 5' end to a small protein. This explains our previous failure to transfer phosphate with polynucleotide kinase to the poly(U) of phosphatase treated polio minus RNA²⁰.

The absence of terminal triphosphates and the presence of VPg on nascent strands of polio RI is direct evidence not previously reported that synthesis of poliovirus RNA occurs from 5' to 3'. It also suggests that VPg may be involved in an early step of RNA synthesis.

Possible role of VPg in replication

Our previous failure to find pppNp in digests of polio RI suggested to us that initiation does not occur *de novo* but by an alternative mechanism, possibly by a primed reaction (see discussion in ref. 5). The 5'-terminal structure of polio virion RNA, of nascent strands in polio RI, and of polio minus strand RNA, which we report here, implicates a most unusual molecule for the role of a primer in RNA synthesis: a small, basic protein. Our current view of the role of that protein, VPg, in replication

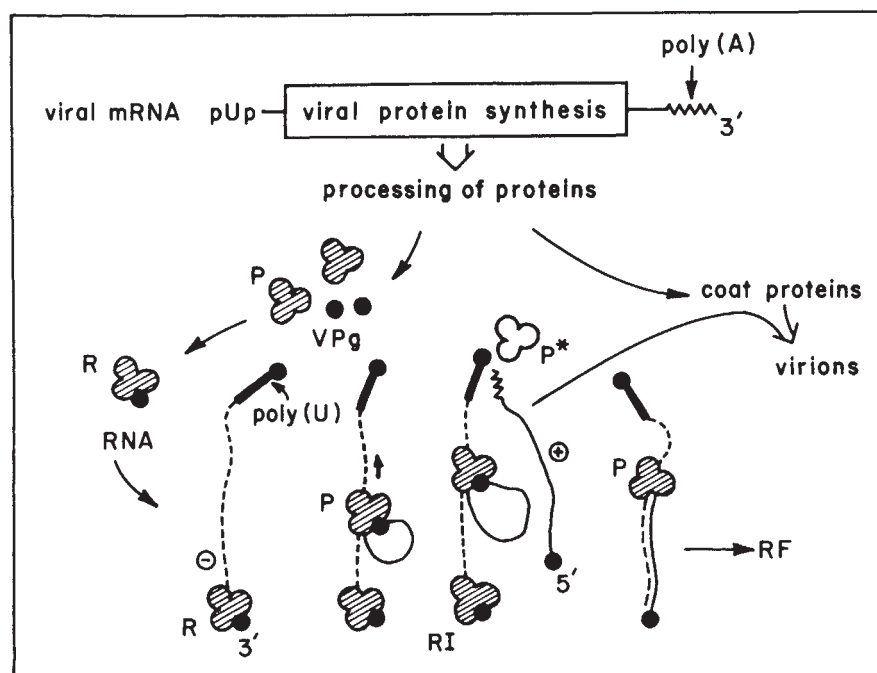


Fig. 6 Involvement of the genome-linked protein VPg in polio virus RNA synthesis. The model shows only synthesis of plus strands but applies also to synthesis of minus strands. RI, replicative intermediate; RF, replicative form (double stranded RNA); R, replicase; P, polymerase.

is illustrated in Fig. 6. Following the post-translational cleavage of viral proteins (see ref. 21 for review), newly made VPg associates with newly made protein P (a hypothetical, virus-specific RNA polymerase) to form the replicase R. Replicase R, but not polymerase P, is able to initiate plus or minus strand synthesis whereby VPg is linked to the nascent RNA. Strand elongation will then proceed during which the RNA-linked VPg remains attached to the polymerase. This condition would prevent the nascent strand from annealing to the complementary template RNA. After the transcription of RNA and its 3'-terminal poly(A) (ref. 22) has been completed, progeny strands and polymerase P* are released. The released polymerase P* may or may not be competent to reassociate with free VPg and re-enter the replicative cycle. It should be stressed that VPg could enter the replication complex also in form of the peptide chain of a precursor protein. Initiation of RNA synthesis may then occur concomitantly with proteolytic cleavage of the precursor yielding a nucleotidyl-protein.

Should the RNA-linked VPg dissociate from polymerase P after initiation of RNA synthesis, strand elongation will occur in the absence of the 'initiator' protein. As a consequence the nascent strand may anneal to the template RNA thus generating double-stranded RNA, the polio RF. In fact kinetic studies¹⁷ and biochemical studies¹⁹ have shown that polio RF is a by-product of replication and not an artefact of isolation procedures.

A tight coupling of poliovirus RNA synthesis to viral protein synthesis exists throughout the infectious cycle even though the sites of translation and replication are physically separated¹⁷. Our model offers an explanation for this control mechanism: the attachment of protein VPg to RNA requires that VPg be continuously supplied by the translational machinery.

Finally, one striking finding is that the viral mRNA is not identical to virion RNA as previously assumed¹⁷ in that it lacks VPg. Instead, polio mRNA is 5'-terminated with pUp⁵⁻⁷. This requires that VPg be cleaved from infecting virion RNA. To what extent this type of 'processing' occurs—cleavage of just the

protein or of an oligonucleotidyl-protein—will be known when we have identified the 5'-terminal nucleotide sequence of viral mRNA. Removal of the positively charged protein could be obligatory for translation and might, at the same time, render viral mRNA incompetent for encapsidation. In fact, mRNA is not a precursor for virions¹⁷ which might implicate VPg in the morphogenesis of the virion. It should be mentioned that the protein is not essential for virion RNA to be infectious since RNA from which VPg has been removed proteolytically fully retains its specific infectivity (Golini, A. N. and E. W., unpublished).

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Purification and structural characterisation of human *HLA*-linked B-cell antigens

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The human B cell-specific alloantigen which is closely linked genetically to HLA contains two non-covalently associated, sialoglycoprotein subunits of molecular weight (MW) 29,000 (p29), and 34,000 (p34). Although p29 and p34 have different amino-terminal sequences, their tyrosine peptide maps indicate considerable similarity in other portions of their polypeptide chains. Thus the genes for their proteins may have evolved by duplication of a common ancestral gene. Another lymphocyte cell surface protein of MW 16,000 (p16) has also been characterised. Both p16 and p44 (the heavy chain of HLA-A,B antigens) have been compared with p29 and p34.

A HUMAN system of cell surface alloantigens has recently been identified which is expressed predominantly on B lymphocytes, and is determined by a gene in *HLA* (refs 1-4), the major histocompatibility complex (MHC) of

humans. At least two genetic loci for B-cell alloantigens have been identified in the *HLA* region separable by crossing over⁵. One of these loci is either identical to or in very strong linkage disequilibrium with the *HLA-D* locus, the genetic locus causing the strongest stimulation of the mixed lymphocyte culture (MLC) (ref. 1). Human *HLA*-linked B-cell antigens are serologically distinct from the cell surface antigens which are products of the classical *HLA-A* and *HLA-B* loci. It has previously been shown that papain solubilised and purified *HLA*-linked B-cell alloantigens consist of a complex of two polypeptides of 23,000 (p23) and 30,000 (p30) molecular weight (MW) (refs 6-8). Antisera raised against this complex are potent inhibitors of the MLC reaction and precipitate polypeptides of 29,000 and 34,000 MW (refs 6-9) from detergent-solubilised extracts.

This report describes the isolation of detergent soluble *HLA*-linked B-cell alloantigens, analysis of their subunit structure, and characterisation of the subunits by amino acid analysis, NH₂-terminal sequencing, and peptide mapping.

Purification

Membranes were prepared from RPMI 4265 lymphoblastoid cells, solubilised with the non-ionic detergent Brij 99 : Brij 97 (2 : 1), and after high speed centrifugation, the detergent soluble supernatant was adsorbed on to and eluted from a *Lens culinaris* lectin affinity column^{10,11}. The eluate was subjected to gel filtration on a BioGel A-5m column (Fig. 1). Fractions from the area of the column containing protein were analysed by sodium dodecyl sulphate (SDS) gel electrophoresis (Fig. 1, inset) and placed in pools A, B and C. The material applied to the BioGel A-5m column consisted predominantly of six polypeptides of MW 105,000 (p105), 44,000 (p44), 34,000 (p34), 29,000 (p29), 16,000 (p16), and 12,000 (p12). p105 and p16 were in a sharp peak at 900,000 MW (pool A), and p44 and p12, the subunits of HLA-A,B antigens, were in a sharp peak at 400,000 MW (pool C) with some aggregated p44,12 at higher MW. p29 and p34, which are the subunits of the HLA-linked B cell alloantigen (see below), chromatographed at an intermediate MW in a broad peak indicative of partial aggregation (pools A and B) and were largely separated from p44,12. The fact that the ratio of p29 to p34 was constant in all fractions of the column was consistent with non-covalent association between p29 and p34. Membrane suspensions were prepared in the presence or absence of 1 mM dithiothreitol (DDT) (refs 13, 14). The preparations with no DDT were somewhat less pure, but p29 and p34 showed a similar elution pattern during BioGel A-5m filtration.

Subunit structure

After preparation in the absence of DDT, the fractions from BioGel A-5m filtration enriched in p29 and p34 were incubated with 0.6% SDS at various temperatures, in the presence of iodoacetamide (IAA) or 2-mercaptoethanol (ME) and electrophoresed on SDS gels (Fig. 2a, b). Comparison of the patterns at different temperatures showed that the HLA-A,B antigen subunits, p44 and p12, were dissociated and electrophoresed identically after incubation

at all temperatures. But, after incubation at 21, 27 or 38 °C in SDS, p29 and p34 were associated in a complex (p29,34) at an apparent MW of 55,000, but were dissociated at 100 °C. Interestingly, when pooled human IgG was similarly treated with ME and SDS at 21 °C, L chains were dissociated, but H chains remained associated non-covalently (at the ME concentration used¹⁵) in an H₂ dimer which calibrated at 90,000 MW (Fig. 2d). Since dissociation into p29 and p34 occurred at 100 °C in the presence of 10 mM iodoacetamide to prevent disulphide interchange (Fig. 2a), it may be concluded that p29 and p34 are not linked by disulphide bonds. The mobilities of the subunits in reduced gels (Fig. 2b) were different from those in unreduced gels (Fig. 2a), particularly in the case of p29. The identity of reduced and unreduced bands was confirmed by cutting out bands from unreduced gels, reducing and electrophoresing them. Two dimensional SDS gels of the same preparation using electrophoresis without reduction in the first dimension, followed by reduction and electrophoresis in the second dimension, also showed that while p44 disulphide-linked dimers were present in these preparations, p29 and p34 were not disulphide-linked (see Fig. 7 in ref. 13 and Fig. 2 in ref. 14). But, cross-linking experiments with two cleavable cross-linking reagents containing S-S bonds^{16,17} and isoelectric focusing showed that p29 and p34 are non-covalently associated with each other and that dimers of p29 alone or of p34 alone are not present (see Figs 3 and 4 in ref. 18).

Purified p29,34 was prepared by incubation of pool B in SDS at room temperature, followed by preparative SDS gel electrophoresis. Immunisation of rabbits with this material elicited a B cell specific antiserum with properties much like the B cell specific anti-p23,30 heteroantisera reported previously (refs 6-8, 18; T.A.S., J.F.K. and A. Fuks, in preparation). Moreover, purified p29,34 antigen specifically inhibited an HLA-linked B cell alloantiserum Po⁴¹. Heat-dissociated p29 and p34 did not elicit a detectable antibody response in rabbits and did not inhibit either the human alloantiserum or the rabbit heteroantiserum prepared against the p29,34 complex.

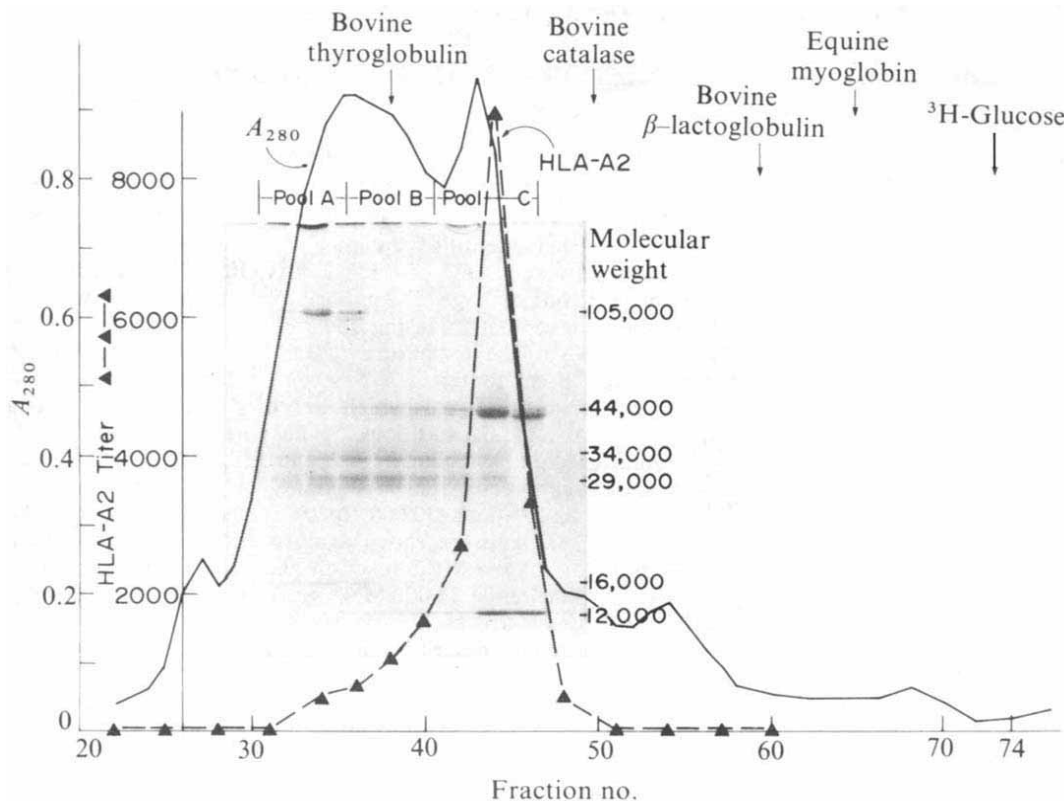
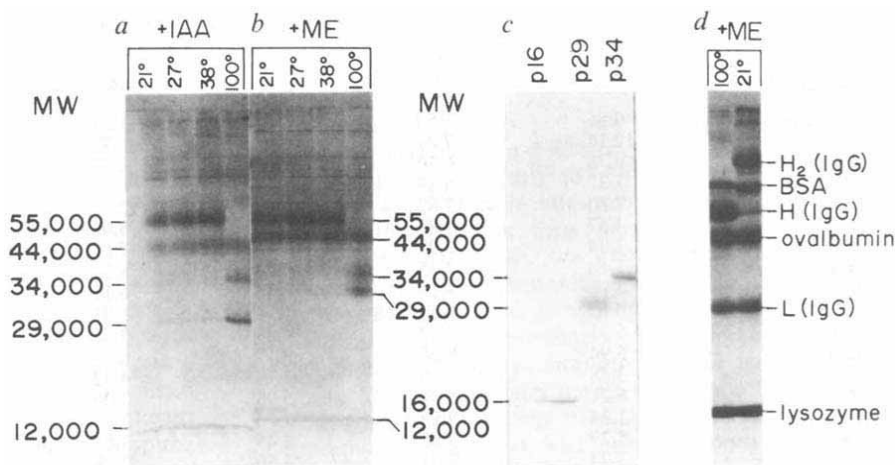


Fig. 1 BioGel A-5m filtration and SDS gel electrophoresis profile of p29,34. BioGel A-5m filtration of the lectin column eluate was as described^{10,11}. Equal volumes of the even numbered fractions 30-48 were precipitated with five volumes of acetone and electrophoresed on Laemmli 11% polyacrylamide-SDS slab gels¹², after boiling in 5% ME sample buffer, and in the figure are aligned with their respective BioGel fractions.

Fig. 2 SDS gels of p29,34 preparations after various treatments. *a*, SDS gel electrophoresis in 10% acrylamide Laemmli gels¹² of material purified in the absence of DTT by lectin affinity chromatography and gel filtration^{13,14} after incubation with 0.6% SDS and 10 mM IAA for $\frac{1}{2}$ h at 20, 27 and 38 °C or for 5 min at 100 °C. *b*, As above, except incubation was in the presence of 5% ME instead of IAA. *c*, 2 μ g of each polypeptide eluted from gels¹⁹ was electrophoresed in an SDS7–15% acrylamide gradient gel. *d*, A mixture of standards (IgG, bovine serum albumin (BSA), ovalbumin, and lysozyme) were electrophoresed after treatment as in (*b*).



Chemical characterisation

The subunits of the *HLA*-linked B-cell alloantigen, p29 and p34, as well as p16, an unrelated protein which also bound to *Lens culinaris* lectin affinity columns and was obtained in good yield, were chemically characterised.

Materials in pools A and B were combined, alkylated with 3 mM iodoacetic acid and heated at 100 °C in the presence of SDS. Proteins were then purified by preparative electrophoresis in 0.8×22 cm, 10% polyacrylamide tube gels using precautions described by Weiner *et al.*¹⁹. Advantage was taken of the greater separation of p29 and p34 in the unreduced state (see Fig. 2*a*). After elution, the proteins were chromatographed twice on a 0.9×40-cm Sephadex G-50 column equilibrated with 20 mM NH_4HCO_3 to remove SDS and substances which interfered with the Edman degradation, and were lyophilised for amino acid and NH_2 -terminal sequence analysis. Equimolar amounts of p29 and p34 were recovered, as measured by either the Lowry assay²⁰ or amino acid analysis. Analytical electrophoresis of the eluted polypeptides (Fig. 2*c*) showed them to be homogeneous.

The peptides were also shown to be homogeneous by amino-terminal analysis using the SDS-dansyl technique¹⁹ and by automatic amino-terminal sequencing (Table 1). The amino-terminal sequences of p29 and p34 are quite different from one another, and from p16. Strikingly, the NH_2 -terminal amino acids of p29 and p34 are identical to those of p44 and p12 (refs 11, 21, 22) although no further homologies are obvious. Much more extensive sequence data for all of these polypeptides will be required to examine the possibility of homology among them.

The amino acid compositions of p16, p29 and p34 were determined by performing hydrolysis for 24, 48 and 72 h and applying samples at two concentrations to a Beckman 121 M analyser equipped with a microbore single column system (Table 2). The composition of p44 from detergent soluble *HLA*-A,B antigens (a mixture of three specific-

ities)^{10,11} is included in Table 2 for comparison. The amino acid compositions of p29 and p34 are strikingly similar, suggesting that these proteins may be related. The amino acid compositions showed that p29 and p34 do not contain an unusually low percentage of polar residues, suggesting that only a short segment of their polypeptides is involved in membrane binding. This is consistent with the finding that papain-solubilised *HLA*-linked B cell alloantigens (p23,30) contain polypeptides of only slightly shortened length⁸⁻⁹.

The sialic acid content (Table 2) was 2.2 and 2.6 mol per mol for p29 and p34 respectively, and thus they are both sialoglycoproteins with amounts of sialic acid similar to that of *HLA*-A,B antigen heavy chains²¹. Staining of SDS gels with periodic acid-Schiff reagent (PAS) also showed that p29 and p34 are glycoproteins. Since glycan moieties which have affinity for the *Lens culinaris* lectin are generally linked by a glycosylamine bond to asparagine and are branched oligosaccharides with galactosyl-*N*-acetylglucosamine sequences in the outer chains and mannose residues in the core²⁶, the carbohydrate compositions of p29 and p34 may be similar to that of p44 (ref. 21). p16 contained 0.5 mole sialic acid per mol, but PAS staining was not detected.

Since p29 and p34 are glycoproteins, their true MWs may be lower than the MW estimated by SDS gel electrophoresis, as is the case for the heavy chain of *HLA*-A,B antigens²¹. Using these molecular weights as a starting point, the peptide MWs of p29, p34 and p16 were calculated from the amino acid compositions²⁷ and found to be 23,600; 28,400; and 15,600 respectively, and the latter MWs were used for calculation of amino acid composition in residues per molecule (Table 2). Three cysteines were found in p29 and p34 (although cysteine may have been underestimated since p12 contained 0.8 residue per mol). p29 and p34 which had been treated with 1 mM DTT and 3 mM iodoacetic acid in the absence of SDS in order to alkylate easily reduced S-S bridges and free SH groups, were also analysed. They contained 0.8 and 1.0 residue of

Table 1 Amino terminal sequences

	1	2	3	4	5	6	7	Residue 8	9	10	11	12	13	14	15
p16	Thr	Glu	Thr	[Thr]		Phe				Lys	Phe		Pro	Asp	
p29	Gly	Asp	Thr	Pro			Phe	Leu	Glu	Gln	Val				
p34	Ile	Lys	Glu	Glu	[Arg]	Val	Ile		Gln	Ala	Glu	Phe	Tyr	Leu	Asn

Peptides (10–30 nmol) were sequenced using ³⁵S-phenylisothiocyanate (New England Nuclear) in an updated Beckman 890 B Sequencer²¹. Phenylthiohydantoin were identified after thin-layer chromatography on polyamide sheets by visual inspection and autoradiography. Arginine, lysine, isoleucine and leucine were identified on the amino acid analyser after back hydrolysis. Peptides were also sequenced using the SDS-Edman-dansyl technique¹⁹.

Table 2 Amino acid and sialic acid composition

Amino acid	mol per 100 mol				residues per molecule		
	p16	p29	p34	p44**	p16	p29	p34
CM-Cys	0.55	1.50	1.21	1.31	0.8(1)	3.1(3)	3.0(3)
Asp	12.14	7.87	8.93	8.73	17.0(17)	16.2(16)	22.3(22)
Thr*	10.78	6.70	6.65	6.87	15.1(15)	13.8(14)	16.6(17)
Ser*	7.32	6.87	5.32	6.77	10.2(10)	14.2(14)	13.2(13)
Glu	7.80	12.62	11.61	12.65	10.9(11)	26.0(26)	29.0(29)
Pro	4.37	4.82	6.26	5.06	6.1(6)	9.9(10)	15.6(16)
Gly	7.04	7.93	6.89	7.67	9.9(10)	16.4(16)	17.2(17)
Ala	7.16	4.85	5.27	8.32	10.0(10)	10.0(10)	13.1(13)
Val†	7.90	8.14	8.48	6.19	11.1(11)	16.8(17)	21.1(21)
Met	0.63	1.52	1.46	1.51	0.9(1)	3.1(3)	3.6(4)
Ile†	5.31	3.33	4.89	3.45	7.4(7)	6.9(7)	12.2(12)
Leu	5.94	8.56	9.17	6.94	8.3(8)	17.7(18)	22.9(23)
Tyr	3.58	3.76	2.73	4.32	5.0(5)	7.8(8)	6.8(7)
Phe	6.21	5.31	5.98	3.06	8.7(9)	10.9(11)	14.9(15)
His	1.34	2.83	2.70	2.91	1.9(2)	5.8(6)	6.7(7)
Lys	5.79	3.88	4.45	3.87	8.1(8)	8.0(8)	11.1(11)
Arg	3.24	7.01	5.56	7.19	4.6(5)	14.5(14)	13.8(14)
Trp‡	2.90	2.51	2.44	(3.2)¶	4.1(4)	5.2(5)	6.1(6)
Polarity §	48.4	47.8	45.3	49.0			
Sialic acid					0.5	2.2	2.6

Values in parentheses rounded to the nearest integer.

* Extrapolated to zero time values.

† 72-h values.

‡ Determined by using a modification of the method of Edelhoch (23) in 0.01% SDS.

§ Calculated according to ref. 24.

|| Determined by the method of Warren (25).

¶ Assumed to be the same as for the papain product (21).

** From refs 10 and 11.

carboxymethyl-cysteine per mol respectively. The additional cysteines of p29 and p34 were either in intrachain disulphide bridges or unreactive. The fact that the mobilities of p29 and p34 in SDS gels (Fig. 2a, b) are slower after reduction suggests that they each contain at least one intrachain disulphide bridge. Even during purification in the absence of DDT, as shown above the easily alkylated cysteines are not oxidised to form interchain disulphide bridges between p29 and p34.

Subunit homologies in tryptic peptide maps

Although limited NH₂-terminal sequencing did not reveal homology between p29, p34 and p44, homologies between polypeptides of dissimilar size do not necessarily begin at the NH₂-terminus. Since the amino acid compositions suggested such an homology, these proteins were compared by tyrosyl peptide mapping.

Carboxymethylated p16, p29, p34 and p44 were heated at

100 °C in the presence of SDS and labelled with ¹²⁵I. After removal of SDS, they were digested with trypsin²⁸. Comparison of the ¹²⁵I-tyrosine tryptic peptides by one-dimensional silica-gel chromatography (Fig. 3a) showed that p29 and p34 had an almost identical peptide pattern. Spots due to free iodine, present in all samples, migrated close to the solvent front in the one dimensional system (and moved out of the area shown on the two dimensional maps illustrated in Fig. 3b). Two-dimensional maps (Fig. 3b) showed that p29 and p34 shared six major ¹²⁵I-tyrosine tryptic peptides (labelled A and B). p29 and p34 also each seem to contain unique peptides (labelled C and D) which agrees well with amino acid analysis showing p29 and p34 to contain eight and seven tyrosines, respectively. p44 showed considerable differences but seemed to contain one peptide (A) in the same position as a peptide of both p29 and p34. No significance should be attached to this single similarity without much further study. p16 showed no homology with

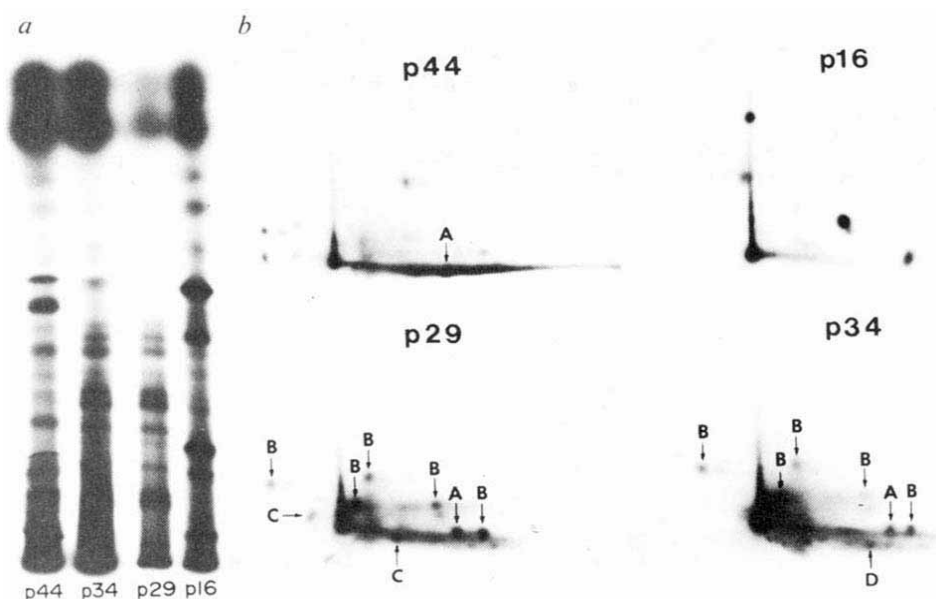


Fig. 3 ¹²⁵I-tyrosine-labelled tryptic peptide maps of p16, p29, p34 and p44. Peptides were labelled with ¹²⁵I in the presence of SDS, digested with trypsin, and separated on silica gel G (ref. 28). HLA antigens were prepared as described in refs. 10 and 11 and p44 was separated from p12 by preparative SDS gel electrophoresis¹⁹. a, Chromatography dimension only. b, Chromatography (upward) followed by electrophoresis (anode to right). The origin is the intensely radioactive spot near the lower left corner. Labelled spots: A, peptides common to p29, p34 and p44; B, peptides common to p29 and p34; C, peptides unique to p29; D, peptide unique to p34.

any of the other peptides and provided evidence that none of the homologies observed in two-dimensional maps were due to artefacts. Thus p29 and p34 are highly homologous to one another. p29 and p34 could not be proteolytic products of p44, since four peptides not present in p44 were found in p29 and p34. Several pieces of evidence show that p29 is not a proteolytic breakdown product of p34. p29 contained one more serine and tyrosine than p34 (Table 2) and two unique tyrosine tryptic peptides (Fig. 3). Additionally, p34 contained a tyrosine residue at position 13 (Table 1), and therefore, proteolytic cleavage from the NH₂-terminus would have produced a product containing two fewer tyrosines than actually found in p29. Proteolysis of p29 and p34 during preparation or storage was never noted, and the ratio of the two polypeptides always remained constant, as determined by SDS gel electrophoresis. Equimolar amounts of p29 and p34 were recovered after preparative SDS gel electrophoresis. Proteolysis would have been expected to result in a mixture of molecules of (p34)₂, (p29)₂ and (p29)₁(p34)₁ structures, but only molecules of the latter structure were found in cross-linking experiments. p29 and p34 each also seemed to be uniquely cleaved by papain digestion, since one polypeptide was converted to p23 and the other to p30. Recent studies of somatic hybrids have also suggested that p29 and p34 are products of different genes²⁹.

Two likely explanations for the high degree of homology between p29 and p34 remain. A common portion could be encoded by one gene, and unique portions, probably at the NH₂-terminus could be encoded by different genes (a two gene, one polypeptide model, as in antibodies). Alternatively, the genes for p29 and p34 could have arisen by duplication of a common ancestral gene. Since this is the case for two other genes in *HLA*, *HLA-A* and *HLA-B* (ref. 21), this seems a likely explanation. Inhibition of the alloantiserum suggests that at least one of the p29 and p34 subunits is a product of one of the *HLA* gene loci which code for B cell alloantigens. But, since genes which code for homologous subunits are often unlinked (as is the case for the immunoglobulin L and H chain genes³⁰), p29 and p34 would not necessarily both be products of the *HLA* region.

If the tyrosine tryptic peptides are representative of the remaining three-quarters of the tryptic peptides of p29 and p34, these molecules would be identical throughout most of their sequences. It will be interesting to determine by further structural analysis whether non-covalent association occurs between homologous regions, and whether differences between the subunits are confined to the NH₂-terminal region. Since RPMI 4265 cells may be heterozygous, and in analogy to the mouse *I* subregions³¹, closely linked loci might specify chemically similar yet distinct molecules, p29 and p34 preparations may well contain chemically different populations of molecules, which might explain the electrophoresis of p29 as a doublet in high resolution Laemmli SDS gels (Fig. 1). Such differences must be limited, however, as no obvious heterogeneity was detected by NH₂-terminal sequencing and close to the expected number of major tyrosine tryptic peptides was obtained.

Relationship to murine and guinea pig Ia antigens

Genetic and serological studies have demonstrated the following similarities between murine Ia antigens and human *HLA*-linked B-cell alloantigens. (1) There genes are in the MHC and in intimate association with MLC genes^{1,31}; (2) Antisera to these products are potent inhibitors of the MLC reaction^{6,7,31} and also block generation of plaque-forming cells *in vitro*³². And (3), they have similar tissue

distributions^{1-9,31}. Our report shows that the two-chain, glycoprotein structure of *HLA*-linked B-cell alloantigens seems similar to that of murine and guinea pig Ia antigens^{28,31,33}. Moreover, the NH₂-terminal sequence of the guinea pig Ia antigen smaller chain³³ is identical to that of human p29 in three out of seven positions, strongly suggesting inter-species homology. Similarly, sequence studies have shown that *HLA-A* and *HLA-B* antigens are homologous to murine H-2 D and K antigens³⁴ and to guinea pig GP-LA B antigens³³. However, murine and guinea-pig Ia antigens analysed after immunoprecipitation have been found on some but not other occasions to contain a disulphide bridge between their subunits^{33,35}. Thus, these rodent Ia antigens might also contain free cysteines in each subunit, but would differ from the human analogues studied here in that their sulphhydryls can readily form an interchain disulphide link.

It is not yet known whether *HLA*-linked B cell alloantigens resemble murine and guinea pig antigens in a further respect. The latter alloantigens are termed immune associated (Ia) because their genes co-map with (but are not necessarily identical to) immune response (*Ir*) genes³¹. This type of close association has not been established in humans. However, susceptibility to certain diseases, which may reflect the absence of particular *Ir* genes, is linked to the *HLA* region³⁶.

Ir gene function may be expressed in T cells, B cells, or both³⁷⁻³⁹. Stimulated T cells express Ia antigens, although in lower amounts than B cells^{39,40}. It is thus possible, although not yet demonstrated, that Ia antigens may in fact be the products of *Ir* genes. If so, the homologies between the subunits of *HLA*-linked B-cell alloantigens would have implications for its role in the immune system as a human *Ir* gene product. Proteins with homologous subunits (immunoglobulins, haemoglobins, and isozymes are examples) often can be composed of any of a set of interchangeable subunits which are specified by duplicated genes. Since many unique combinations can be made using a much smaller number of unique subunits, great diversity in functional specificity can be generated in an economical fashion.

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letters to nature

Water vapour concentrations at the mesopause

MEASUREMENTS of atmospheric water vapour concentrations have been confined to altitudes below about 50 km showing an average volume mixing ratio of about 4×10^{-6} in the middle and upper stratosphere¹. Only limited and rather indirect observational information on water vapour exists above 50 km including, for example, the presence of hydrated positive and negative ions and noctilucent clouds likely being composed of water ice particles. In a previous attempt to derive water vapour concentrations from measurements of hydrated positive ions, a mixing ratio between 1 and 2×10^{-6} below 78 km and a sharp decrease above was inferred². In this paper, water vapour concentrations are derived at heights between 85 km and 94 km from two rocket-borne mass spectrometer measurements of atmospheric positive ions. The experiments took place at Andoya (69°N, 16°E) on May 31 1972 (ref. 3) and at Kiruna (68°N, 21°E) on August 18 1974 (ref. 4).

The method applied is based on the atmospheric ion reactions



The reaction coefficients $k_2 = 1.9 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ (ref. 5) and $k_3 = 3.2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ (ref. 6) are known from laboratory measurements. They are expected to have no significant temperature dependence. Although no laboratory value exists for k_1 , it is reasonable to adopt a value of $1.5 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$, as most switching reactions have rate coefficients between 1 and $2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ (ref. 7). In the conditions considered (high electron densities in excess of 10^5 cm^{-3}) the dominating loss process for $\text{NO}^+ \cdot \text{H}_2\text{O}$ and $\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}$ was electron recombination. The recombination coefficient for $\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}$ is $\alpha_2 = 3 \times 10^{-6} (300/T)^{0.5} \text{ cm}^3 \text{ s}^{-1}$, (ref. 8) whereas for $\text{NO}^+ \cdot \text{H}_2\text{O}$ an $\alpha_1 = 2 \times 10^{-6} (300/T)^{0.5} \text{ cm}^3 \text{ s}^{-1}$ is adopted according to a systematics of α values⁹. Other loss processes for $\text{NO}^+ \cdot \text{H}_2\text{O}$ and $\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}$ can be neglected, as no ions other than NO^+ and O_2^+ were found in significant abundances besides $\text{NO}^+ \cdot \text{CO}_2$, $\text{NO}^+ \cdot \text{H}_2\text{O}$, and $\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}$. Processes decomposing $\text{NO}^+ \cdot \text{H}_2\text{O}$ to form NO^+ , like photodestruction¹⁰ or thermal decomposition, are much slower than electron recombination. Instrumental effects like electric field or shock-induced collisional decomposition of the ions considered, can also be neglected as in these experiments cluster ions of much lower bond energies were measured between 85 km and stratospheric altitudes.

Assuming chemical equilibrium the continuity equations for $\text{NO}^+ \cdot \text{H}_2\text{O}$ and $\text{H}_3\text{O}^+ \cdot \text{OH}$

$$[\text{NO}^+ \cdot \text{CO}_2] k_1 [\text{H}_2\text{O}] = [\text{NO}^+ \cdot \text{H}_2\text{O}] \alpha_1 [e] \quad (4)$$

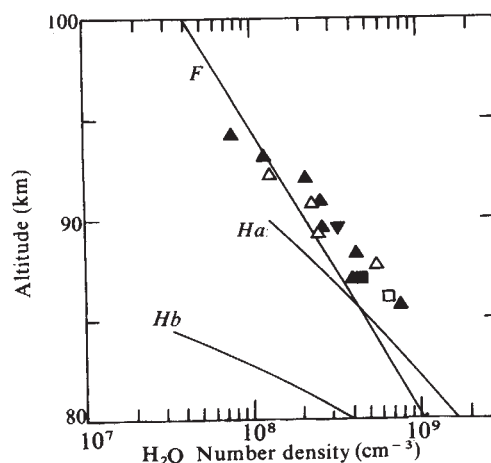
$$[\text{O}_2^+ \cdot \text{H}_2\text{O}] k_2 [\text{H}_2\text{O}] = [\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}] \alpha_2 [e] \quad (5)$$

relate the water vapour number densities to the measured ion

densities, the measured electron density, and the reaction and recombination coefficients. Equation (5) neglects electron recombination of the intermediate ion $\text{H}_3\text{O}^+ \cdot \text{OH}$ as reaction (3) is much faster. The electron density was measured by wave propagation as well as electrostatic probe experiments¹¹ with an accuracy better than $\pm 20\%$. The error of the derived H_2O number densities is mainly introduced by the uncertainty of k_1 and k_2 and by the uncertain temperature dependence of α_1 and α_2 . Relative H_2O -densities are not affected by changes in k_1 and k_2 and are only little affected by changes in the temperature dependence of α_1 and α_2 even if a maximum temperature dependence of $T^{-1.5}$ ¹² is considered. The latter is true because height variations of atmospheric temperatures in the height range considered are relatively small. Regarding absolute H_2O -densities, an increase by as much as a factor of 2.6 has to be considered if a maximum temperature dependence of α_1 , and α_2 of $T^{-1.5}$ is allowed for.

The water vapour number densities derived by use of equations (4) and (5) assuming atmospheric temperatures of the CIRA 1972 model atmospheres are shown in Fig. 1. For each flight both methods gave consistent densities. When compared with each other the H_2O density profiles of both flights agree closely decreasing from $8 \times 10^8 \text{ cm}^{-3}$ at 85 km to $8 \times 10^7 \text{ cm}^{-3}$ at 94 km. The experimental densities are slightly higher than those predicted by photochemical models including strong vertical eddy diffusion (Ha^{13} and F^{14}), but considerably higher than theoretical values based on weak vertical diffusion (Hb^{13}). Using the measured H_2O densities and total gas densities from the CIRA 1972 (ref. 15) model atmosphere for 70° latitude in June and August volume mixing ratios were derived (Fig. 2). They range from 3.5 to 6×10^{-6} for the first and from 3 to 4×10^{-6} for the second experiment. The corresponding average values are 4.5×10^{-6} and 3.5×10^{-6} with no indication for a significant change in the height range 85 km to 94 km. These

Fig. 1 Number densities of water vapour for the experiments F33 (■●) and F36 (□△) derived by the use of equations (4) (▲△) and (5) (■□). Theoretical profiles considering strong (Ha^{13} , F^{14}) and weak vertical turbulent transport are shown for comparison.



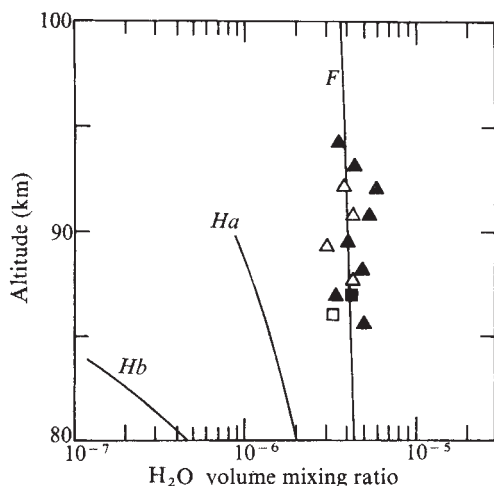


Fig. 2 Water vapour volume mixing ratios (for clarification Fig. 1 caption).

mixing ratios are compatible with the average value of 4×10^{-6} measured in the middle and upper stratosphere¹. They are also close to a model profile considering strong vertical turbulent transport¹⁴. It may, therefore, be concluded that strong vertical transport must have been operative when our measurements were made. The transport mechanism may have been either strong turbulent diffusion or a mean upward motion which has been proposed to exist during summer at high latitudes¹⁶. Further implications from the high H_2O mixing ratios are enhanced hydration of atmospheric ions and enhanced water vapour condensation around nucleation embryos which may be pre-existing solid particles or complex ions. Concerning the ion induced nucleation, a high H_2O mixing ratio is of particular importance as it favours not only the growth of supercritical particles but also the formation of nucleation embryos by subsequent hydration of ions.

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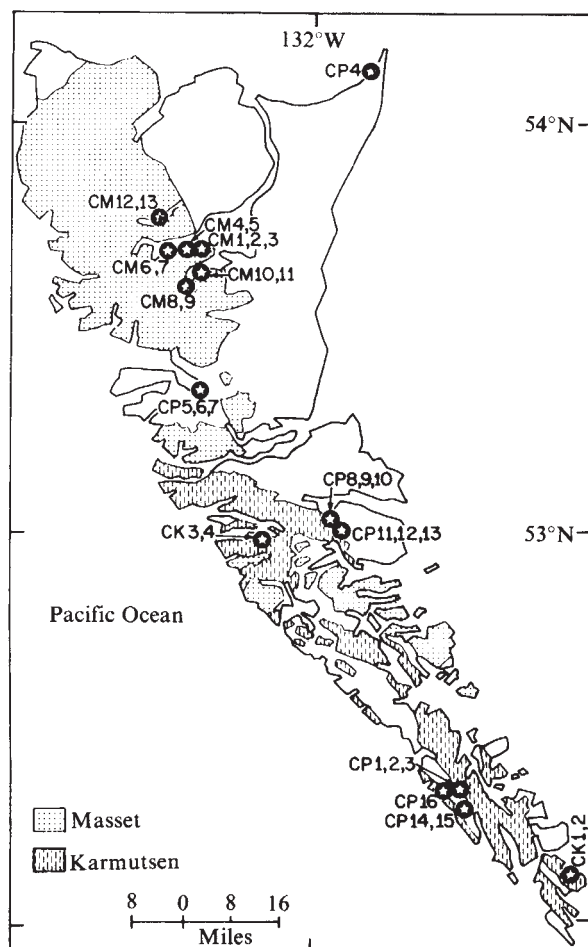
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and only a reconnaissance was possible, but these preliminary results indicate that the islands have rotated clockwise since the Eocene.

The oldest rock unit of the Queen Charlotte Islands is the Late Triassic (Karnian) dominantly volcanic Karmutsen Formation¹. It is overlain by the largely sedimentary Vancouver Group, which is cut by syntectonic foliated batholiths, the Older Plutons. The Older Plutons have K–Ar hornblende ages ranging from 156 to 142 Myr (refs 2–4). The Vancouver Group is unconformably overlain by the dominantly sedimentary rocks of the Long Arm Formation and the Queen Charlotte Island Group. Lying unconformably above these are the volcanics of the Masset Formation which have yielded a K–Ar mica age of 62 ± 6 Myr (ref. 1) indicating a Palaeocene age. The Masset Formation is cut by the post-tectonic Younger Plutons. The Younger Plutons are massive unfoliated bodies, which have yielded K–Ar biotite and hornblende ages ranging from 39 to 26 Myr (ref. 1). This indicates that they are Oligocene, but the ages may represent the time of uplift and the bodies could have been emplaced in the Palaeocene or Eocene. The Masset is overlain unconformably by Neogene sediments. Samples were obtained from the Karmutsen Formation, the Older and Younger Plutons, and the Masset Formation (Fig 1). No coherent magnetisation was found in the Older Plutons (CP1, 2, 3, 14, 15 and 16, Fig. 1).

The Karmutsen Formation is over 4,000 m in thickness and consists mostly of basalts. It was sampled at four sites on Kunghit Island (CK1 and 2), and in Douglas Inlet (CK3 and 4). Excellent coherence after demagnetisation occurs at three sites. At Kunghit the directions are reversed, and at Douglas Inlet they are normal. The rocks have

Fig. 1 Sampling localities in the Queen Charlotte Islands. (Geology from ref. 1.)



Tectonic rotation in Western Canada

THE Queen Charlotte islands of British Columbia form the central part of the Insular Fold Belt, which is the most westerly tectonic unit of the North American Cordillera. In 1971, we collected oriented cores from the islands in an attempt to determine palaeomagnetically their motions relative to the stable craton of North America. A knowledge of these motions is needed before one can understand the origin of the Insular Fold Belt and the Cordillera generally. The localities sampled are difficult to reach,

Table 1 Results of samples taken from the Queen Charlotte Islands

	B*	N	Means	k	α°_{95}
Masset Formation directions	8	52	026, +78	81	6
Masset Formation poles	8	52	097°W, 72°N	24	11
Younger Plutons directions	5	27	338, +80	138	4
Younger Plutons poles	5	27	154°W, 70°N	90	8
Karmutsen Formation directions	3	17	359, +69	68	15
Karmutsen Formation poles	3	17	165°W, 89°N	28	24

*B is the number of sampling sites, N the number of cores (two specimens each), declination (long.) and inclination (lat.) of mean direction (pole) calculated irrespective of sign, k is the precision, and α°_{95} the circle of confidence. All specimens were cleaned in alternating fields of 30–40 mT. (Site locations and statistics may be obtained from E. Irving).

been metamorphosed to greenschist or amphibolite facies during the Late Jurassic, and the magnetisation could be of this age.

One Younger Pluton was sampled between 1 and 2 km north of Louise Narrows. The rocks are massive quartz monzonites. They give excellent results. Three sites are reversed, and two are normal. Five cores of Karmutsen basalt from the contact aureole 30 m north of the contact gave general poor overall coherence, but three other cores had reasonable end points with a mean of 345, +51, in rough agreement with directions just inside the pluton (CP11 and 13). It is difficult to estimate the horizontal plane at the time the plutons were magnetised, but there is no reason to suspect that they have been tilted and no correction has been made.

Thirteen sites were sampled from the Masset Formation on Ian Lake and Masset Inlet (Fig. 1). No coherent

magnetisations were found in the basalts at sites CM1 to 3 and CM12 and 13. At the remaining sites the basalts and rhyolites yielded excellent results. Four sites have normal polarity and four are reversed. Apparent dips of the lavas vary from 0° to 10° and may be depositional so no correction has been made. The mean apparent dip is 5° to the NNW, and application of a correction would not substantially affect our conclusions.

The results are summarised in Table 1. Although the total number of results is not great (16 sites) the precisions are high. The magnetisation of the Masset Formation is considered to be Palaeocene. In Fig. 2 the pole for the Masset Formation is compared with the average pole path for the Cainozoic of a stable North America. The pole is to the SE of its expected position. An anticlockwise rotation of $53 \pm 28^{\circ}$ brings the pole into conformity with the Palaeocene of North America, suggesting that this part of the Queen Charlotte Islands has rotated at least 25° clockwise since the Palaeocene, indicating that the Insular Fold Belt was originally approximately straight. The pole for the Younger Plutons agrees with the expected Palaeocene position. It is possible that their true age is earliest Tertiary, and that the rotation referred to above may have been completed between the extrusion of the Masset volcanics and the cooling of the Younger Plutons. Only the results from the Masset, however, provide good evidence for rotation, the data from the Younger Plutons being insufficient.

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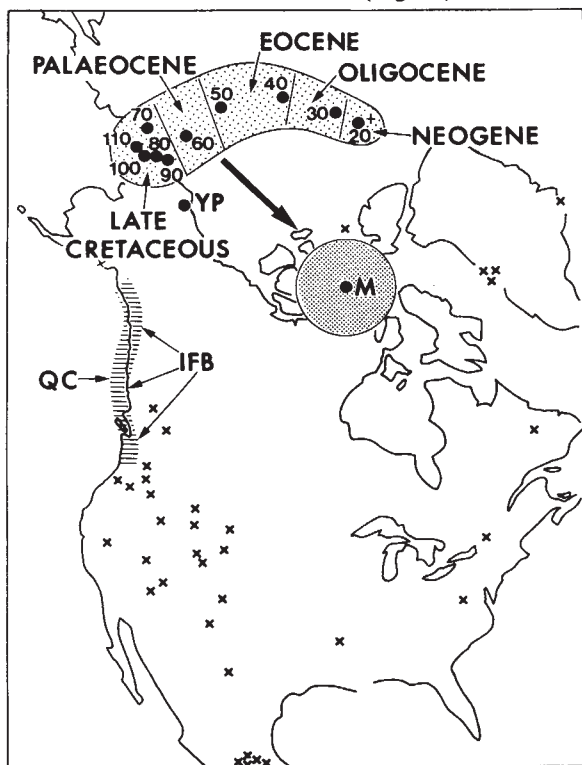


Fig. 2 The pole with its standard error for Masset Formation (M) is compared with the mean polar path for North America. The mean path is based on results obtained from the localities marked X, the results from Greenland being corrected for opening of the Labrador Sea. The mean poles, calculated as overlapping averages for the interval 110 Myr to 5 Myr, are shown, and the zone enclosing them is the envelope of their standard error circles (E. Irving unpublished data.) Standard errors are used because the significance of differences can be readily assessed; if the probable errors do not overlap then the means are significant different at probability of 95%. The present geographical pole is the cross near the Neogene pole, QC, Queen Charlotte Islands, IFB, Insular Fold Belt, YP, pole for the Younger Plutons.

Frequency dependence of elasticity of rock—test of seismic velocity dispersion

LINEAR theories of attenuation of acoustic or seismic waves in media, such as rocks, which are characterised by constant or nearly constant Q factors (fractional energy loss per cycle equals $2\pi/Q$), imply velocity dispersion and, therefore, frequency dependence of elasticity. The effect is small, corresponding to a change of order 1% over the period range of seismological interest (1 s to 1 h), and is consequently difficult to observe. However, it leads to an internal inconsistency in the development of earth models by inversion of free oscillation data and to discrepancies between these models and body wave data^{1–3}. It is a matter of considerable geophysical interest to resolve the problem. Validity of the linearity assumption has been questioned⁴ but we are now observing elliptical hysteresis loops in basalt and granite samples subjected to sinusoidal strains of order 10^{-6} and this is strong evidence that attenuation does become a linear phenomenon at low strain amplitudes. But whether or not perfect linearity applies, a direct demonstration of body wave dispersion is the most satisfying indication that earth model studies need revision.

The requirement of causality in linear attenuation theories (which we propose to review in a separate paper) leads to the relationship between phase velocity v (ω_0) at angular frequency

ω_0 and an integral involving the attenuation coefficient, $\beta(\omega)$

$$\frac{1}{v(\omega_0)} = \frac{1}{c} - \frac{2}{\pi} P \int_0^\infty \frac{\beta(\omega)}{\omega_0^2 - \omega^2} d\omega \quad (1)$$

where P indicates the Cauchy principal value of the integral, c is a constant (phase velocity at $\omega \rightarrow \infty$) and a plane wave of any frequency ω decays with distance x as $\exp(-\beta x)$. For the present purpose, it suffices to assume that the behaviour of β at very high frequencies is such that the integral in equation (1) converges but that over a wide range of frequencies about ω_0 , β is proportional to ω , which is very nearly equivalent to the constant Q assumption because

$$\beta = \omega/2Qv \quad (2)$$

the velocity dispersion being slight. Then it follows that, to first order in Q^{-1} , values of elastic modulus μ (real part of the complex modulus), at frequencies ω_1 and ω_2 , are related by

$$[\mu(\omega_2) - \mu(\omega_1)]/\mu(\omega_1) = (2/\pi Q) \ln(\omega_2/\omega_1) \quad (3)$$

The derivation of equation (3) demonstrates the equivalence for practical purposes of the alternative empirical linear theories⁵⁻⁷, which differ only in the assumptions regarding the high frequency behaviour of β , as well as the mechanistic theory based on logarithmic creep⁸⁻¹¹. The logarithmic dependence of elasticity or velocity on frequency is characteristic of frequency independent Q ; other assumptions regarding $\beta(\omega)$ lead to quite different results, so that the direct observations which we report here, confirming the validity of this relationship, also support the constant Q assumption. The logarithmic dependence seems to have been quoted first by Kolsky¹², but we have been unable to find the earlier derivation. This dispersion law also satisfies the observations of pulse shape in acoustic experiments^{12,13} and in particular the increase of pulse rise time τ with time of travel and Q for the path¹³

$$\Delta\tau \approx 0.5 \int Q^{-1} dt \quad (4)$$

Our experiment is similar in principle to that of McKavanagh and Stacey¹⁴, who measured the mechanical hysteresis of rock cores under cycled axial compressions. Our specimens are tubes of rock of length about 280 mm, diameter 44 mm and wall thickness 5 mm, and are subjected to torsion. The use of thin walled tubes ensures that the strain, which is pure shear, is approximately uniform through the material. The specimen under examination is connected to a standard tube, either of high Q steel or of fused quartz, subjected to the same torque and used to infer the stress. Rings, fixed about 20 mm from both ends of each specimen and standard, are used for mounting capacitance plates, which are centred about 200 mm from the specimens. Relative displacements of the plates record the twist of the specimen between its mounting rings. Balanced sets of plates are mounted on opposite sides of each specimen to minimise effects of asymmetry and thermal drift. Measurements of capacitance are made by means of ratio transformer bridges^{14,15} with rapid response times (10^{-3} s) which avoid the appearance of any instrumental phase lag in the measured strains.

Small stresses are applied by means of loudspeaker coils mounted on a lever arm connected to one end of the standard specimen and driven by current from a low frequency sinusoidal oscillator. Strains in the rock specimen and standard are recorded on an $X-Y$ recorder, giving, in effect, a stress-strain plot as the strain is cycled. With the granite and basalt specimens which we have used in this experiment, the hysteresis loop is barely apparent. However, by subtracting the two bridge outputs from one another (specimen minus standard) in a difference amplifier, with independently controlled gains on the two inputs,

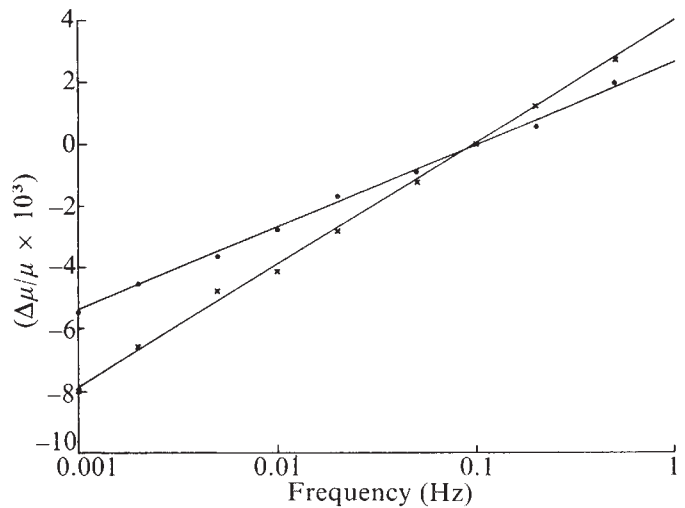


Fig. 1 Frequency dependences of rigidity moduli of basalt and granite specimens over the range 0.001–0.5 Hz relative to values of 0.1 Hz, which was used as a reference frequency in repeated comparisons. ●, Basalt, $Q = 590$, strain amplitude = 2.6×10^{-6} . ×, Granite, $Q = 320$, strain amplitude = 2.9×10^{-6} . By equation (3) the gradients correspond to Q values of 546 and 371 respectively.

and further amplifying the difference signal, we can plot directly the departure of specimen strain from linearity with strain of the standard. This appears as a loop of adjustable width, whose area is a measure of the hysteretic loss in the specimen, relative to the standard. The slope of the axis of this loop is a very sensitive indicator of the elastic modulus. The fact that we are able to observe such fine details of loop shape at strain amplitudes as low as 10^{-6} (hopefully lower with improvements now planned) is due to the high sensitivity and wide linear range of the capacitance displacement transducer¹⁴⁻¹⁷.

We have plotted hysteresis loops for specimens of basalt and granite and found that the elastic moduli vary with frequency precisely in the manner of equation (3) over the period range 2–1,000 s, which covers much of the seismic frequency band. Figure 1 gives plots of the data, represented as the fractional changes in rigidity modulus relative to the values at 0.1 Hz. The gradients of the two graphs do not agree perfectly with the theoretical values obtained from the measured Q values by equation (3). The discrepancies may arise because the standard specimens were not completely lossless or from frequency dependence of Q , although the measured Q values were independent of frequency within the accuracy of our measurements.

The hysteresis loops of both specimens were of elliptical form, not cusped at the ends, indicating that within the precision of these observations the attenuation mechanism is linear. This was not the case with the sandstone specimen examined by McKavanagh and Stacey¹⁴, even down to strains of 10^{-6} , or with granite and basalt specimens at strains of order 3×10^{-6} . It seems that the strain amplitudes used in the present experiment are at the upper limit of the linear range. Further instrumental improvements are required to examine with the same precision Q and modulus changes at strain amplitudes below 10^{-6} .

If we accept the validity of equation (3) then it should be possible to adjust earth free oscillation data by using measured Q 's of the identified modes to remove the effect of frequency dependence of elasticity. Comparison with body wave data also requires recognition of the fact that in the presence of dispersion, as represented by equation (3), the group velocity v_g of a body wave is related to phase velocity by

$$v_g = v(1 + 1/\pi Q) \quad (5)$$

to first order in Q^{-1} . This is the observed velocity in the case of

a body wave, but free oscillations effectively measure phase velocity, which for the mantle is of order 0.1% smaller.

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Carboniferous unconformity in southern Africa

PALYNOLOGICAL investigations indicate that the Dwyka Tillite of southern Africa is probably entirely of Early Permian age and that the Carboniferous System is absent from this region. Early investigations into continental drift¹ indicated that there were identical late Palaeozoic successions in the southern continents. In this letter, this identity is shown to be incorrect in some respects and, as yet, unsubstantiated in others.

The Dwyka Tillite is most fully developed in the Karoo Basin of South Africa (Fig. 1) although occurrences are known as far north as the equator. The unit reaches thicknesses of up to 1,000 m in a deep sedimentary trough which extends along the southern margin of the basin. There are few reliable palaeontological data for the age of the unit. A small marine invertebrate fauna from South-west Africa was dated as Early Permian², and Early Permian miospore assemblages have been reported from Zaïre^{3,4}, Rhodesia⁵, and South-west Africa⁶. A miospore assemblage from above the tillite in the northwestern part of the Karoo Basin was tentatively assigned to the Late Carboniferous⁷, but the forms present could equally well support an Early Permian age. There are no reasons to believe that any part of the unit is younger than Early Permian.

Various ages have been reported for the Dwyka Tillite: Early Carboniferous, possibly Late Devonian, to Early Permian⁸; Late Carboniferous⁴; Early Permian⁹. The principal argument for a Carboniferous component for the age rests on an apparent conformable contact in the southern trough between the Dwyka Tillite and the underlying Waaipoort Formation of Late Devonian or Early Carboniferous age^{8,10}. The Early Permian determinations noted above were all from outside the region of maximum deposition and may represent only later stages.

Within the southern trough, palynomorph microfossils have been badly damaged by heat and tectonism. Three localities (Fig. 1) yielded recognisable specimens. (1) At Elandsvlei, the Dwyka Tillite consists of four sedimentary cycles which can be traced south into the trough and thence eastwards for about 100 km. A sample was taken from the base of the lowest cycle. (2) At Sambokkraal, a deep

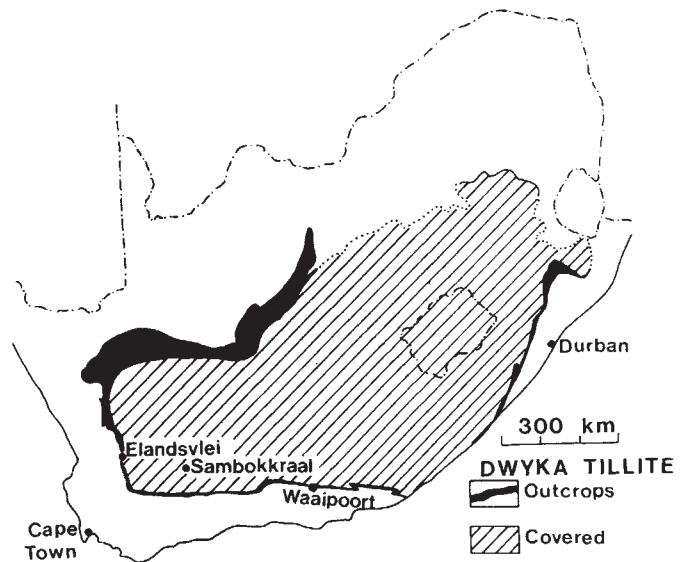


Fig. 1 Distribution of Dwyka Tillite in the Karoo Basin.

borehole penetrated 650 m of Dwyka Tillite. A sample of core was taken 1 m above the base of the unit. (3) At Waaipoort, the Dwyka Tillite unconformably overlies the Kommadagga Subgroup (Fig. 2). A sample was taken from the Miller Diamictite, the lowest unit in the subgroup.

The samples should be uncontaminated. The samples from Elandsvlei and Waaipoort were collected from outcrops and the Sambokkraal borehole was cored throughout. The lithologies are too distinctive to be confused with those of any other units in the region. There is insufficient jointing to permit stratigraphic leakage. Normal precautions against contamination were taken in the laboratory.

All of these samples contained bisaccate pollen grains (Fig. 3a-e). Although the grains were badly damaged, it was possible to determine that some, at least, had striated central bodies (Fig. 3a, b, d). Many large monosaccate grains were also present (Fig. 3f). Early Permian assemblages are characterised by monosaccate and striated bisaccate pollen. The monosaccate grains were too badly preserved to permit identification even to the generic level and similar forms are known from the Late Carboniferous. Striated bisaccate pollen, however, did not appear until well into the Stephanian. Since the sample from the Miller Diamictite was taken 400 m below the Dwyka Tillite, separated from the tillite by an angular unconformity, the base of the tillite is probably above the Permian-Carboniferous boundary. Nevertheless, the possibility exists

Fig. 2 Relationship of Dwyka Tillite to underlying units, southern Karoo Basin.

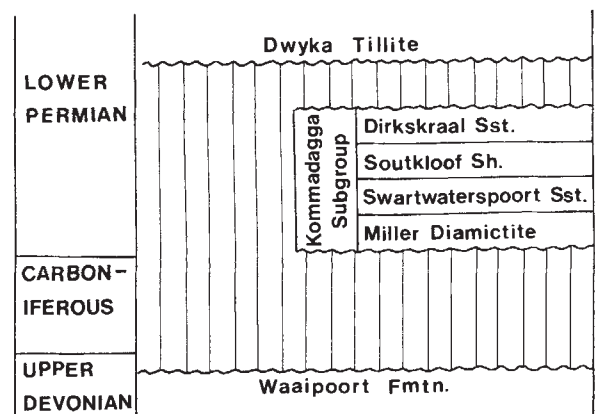
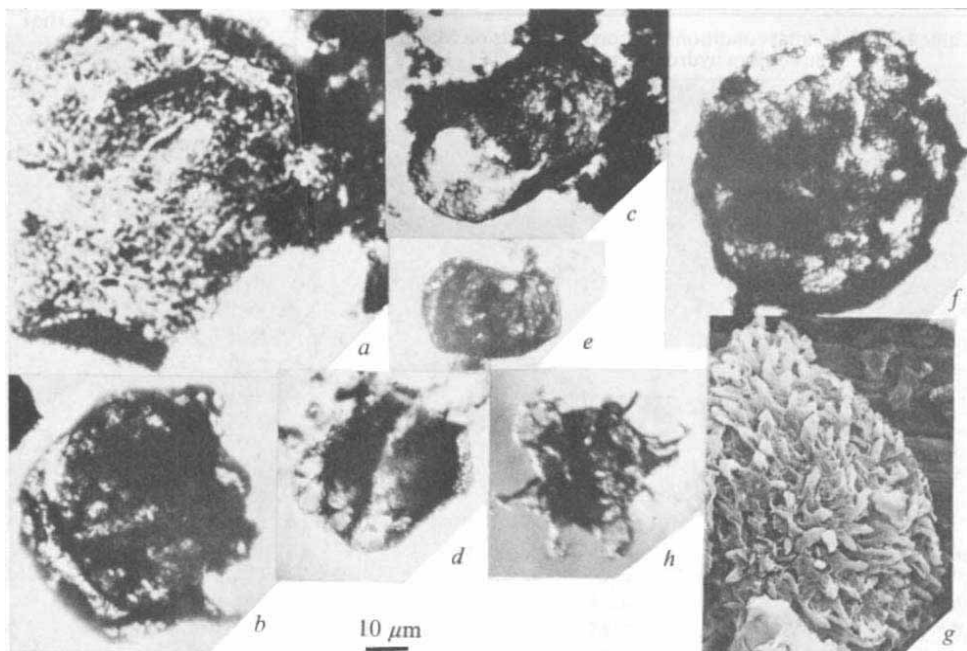


Fig. 3 *a*, Bisaccate pollen, Dwyka Tillite, Elandsvlei. *b*, *c*, Bisaccate pollen, Miller Diamictite, Waaipoort. *d*, *e*, Bisaccate pollen, Dwyka Tillite, Sambokkraal. *f*, Monosaccate grain, Miller Diamictite, Waaipoort. *g*, *Dibolisporites echinaceus*, Waaipoort Formation. *h*, ?Acritarch, Dwyka Tillite, Sambokkraal. Note striations on *a*, *b*, and *d*.



that Dwyka deposition was initiated in the latest Carboniferous.

At Waaipoort, the Kommadagga Subgroup is underlain by the Waaipoort Formation which, further to the west, directly underlies the Dwyka Tillite itself (Fig. 2). Plant megafossils from the Waaipoort Formation have given ambiguous Devonian or Carboniferous ages. Palaeoniscoid fish, from nodules collected about 200 m below the top of the formation, were dated as Visean on the basis of a broad resemblance at the familial level with Carboniferous fish of Britain¹¹. A residue extracted from one of these same nodules contained carbonised Devonian spores including such species as: *Acinosporites acanthomamillatus* Richardson, *Ancyrospora langii* (Taugourdeau-Lanz), *Contagisporites optivus* var. *vorobjevensis* (Chibrikova), and *Dibolisporites echinaceus* (Eisenack) (Fig. 3g). Many of the same Devonian species were found reworked into the Miller Diamictite and some Devonian forms were seen in the Dwyka Tillite at Sambokkraal. The assemblage from the Waaipoort Formation indicates a Givetian or possibly Frasnian age. It is unlikely that the 200 m of the Waaipoort Formation above the sample horizon would extend completely through the Upper Devonian into the Carboniferous. No other Carboniferous rocks have been reported from subequatorial Africa.

There are insufficient data to permit reliable age determinations for the late Palaeozoic diamictites of Antarctica or the Parana Basin of South America. Those of India and Australia are of Early Permian age, with possibly some Late Carboniferous present in both regions. Thus, the Late Palaeozoic diamictites of India, Australia and southern Africa were all deposited within the same time interval, from latest Carboniferous to Early Permian. This interval is too long, and the ages too imprecise, to determine whether or not the units were actually synchronous.

No Carboniferous is known from Antarctica, though this may be due to incomplete exploration. There is Lower Carboniferous in India and fairly complete Carboniferous successions occur in Australia and South America. Thus, the late Palaeozoic history of Gondwanaland is more complex than has hitherto been indicated.

Some possible acritarchs (Fig. 3h) were seen in the Dwyka Tillite at Sambokkraal. No such forms were seen in the Waaipoort Formation and hence they are unlikely to be reworked. It has been postulated that, in the southern trough, the Dwyka Tillite was deposited from a floating

ice shelf¹⁰, which might account for the presence of such forms, but such a model is difficult to reconcile with the presence of gymnosperm pollen in the same sample. These samples were diamictite, not interbedded shale. Pollen has been recovered from Dwyka diamictites in other parts of the Karoo Basin and in Rhodesia³. In the south-western part of the Karoo Basin, the diamictite has an average organic carbon content of about 0.1%, mostly as terrestrial plant fragments. These data seem incongruent with accepted models of sedimentation for the Dwyka Tillite.

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Silicate and the aqueous corrosion of a high-magnesium alloy

FOLLOWING Butler and Mercer's demonstration¹ that silicate derived from dissolution of glass suppresses the corrosion of mild steel in distilled water at 90 °C, we have compared the results of exposure of Magnox AL 80 (a magnesium alloy containing 0.8% of aluminium) to solutions with and without silicate contamination. This showed that silicate enhanced corrosion in alkaline solution, the effect being greater the higher the temperature. Both magnesium hydroxide and an hydrated magnesium aluminium silicate seem to be formed in the corrosion reaction. During our extensive studies² of the aqueous corrosion of Magnox AL 80, care has been taken to store and use alkaline solutions in plastic containers since dissolution of borosilicate apparatus readily causes contamination³. A few tests have, however, been conducted with

Table 1 Experimental conditions for corrosion tests on Magnox AL 80 in sodium hydroxide solutions (pH 11–12)

Test number	Dissolved silicon concentration (mg kg ⁻¹)	Temperature (°C)	Test duration (d)
1	100	60	120
2	0	105	0.17
3	130	105	0.25
4	0	121	0.1
5	130	121	{ 0.1 1.0

solutions known to contain silicate and data from these form the basis of our observations.

Corrosion experiments were carried out using specimens with surface areas of a few cm² cut from Magnox sheet, degreased, and chemically etched in 2% weight/volume citric acid solution for 5 min. The specimens were immersed in sodium hydroxide solutions (prepared from double distilled water) with pH values between 11 and 12. In these solutions magnesium spontaneously forms a passivating film⁴. Conditions of the tests in which the behaviour with and without silicate in solution could be contrasted directly are summarised in Table 1.

In Test 1, specimens were exposed to solution contained in a soft-glass tank, the pH of the liquid being maintained above 11 by occasional addition of fresh hydroxide solution. The dissolved silicon content was variable but averaged about 100 mg kg⁻¹. The solution for Test 5 initially contained 130 mg kg⁻¹ of silicon produced by etching of the flask in which it was prepared. The same silicon concentration in the form of sodium metasilicate was added to uncontaminated hydroxide for Test 3.

Previous measurements over a range of temperatures enabled the corrosion rate in silicate-free solution at 60 °C to be estimated accurately. Measurements at the higher temperatures were made with Magnox samples in PTFE liners within small stainless steel autoclaves each of which was fitted with a pressure transducer and chromel–alumel thermocouple. Average corrosion rates were calculated from the specimens' weight gains, allowing for corrosion product spalling, and assuming the product to be magnesium hydroxide². Independent checks of the overall metal losses derived from the pressure of gas generated in Tests 2, 3 and 5 gave values close to those estimated gravimetrically.

With silicate-free solution our results were consistent with those of Masterson and Harrison⁵ for etched Magnox at 40 °C and with data of Blanchet *et al.*⁶ for the magnesium alloy GA3Z1 between 65 and 95 °C. In comparison,

our tests suggest that corrosion rates for Magnox are a factor of 3 lower than those quoted by Staesche⁷ for a Mg–Mn alloy, although the temperature dependence is very similar. Greenberg and Ruther's experiments⁸ on alloy AZ 31 gave rates approximately twice what we might expect for AL 80.

At 60 °C average corrosion rates of Magnox in solutions containing silicate were indistinguishable from those in uncontaminated solutions, while at 105 °C the average rate was twice as great, and at 121 °C four times higher, than in silicate-free solutions after similar periods of exposure. This enhancement factor for silicate solution had doubled at the end of Test 5. These results contrast with a fivefold decrease in the corrosion rate of mild steel in distilled water at 90 °C produced by 4.7 mg kg⁻¹ of dissolved silicon as reported by Butler and Mercer¹.

On completion of Test 5 the concentration of dissolved silicon had dropped to 0.1 mg kg⁻¹. The possibility that silicate may have reacted with the Magnox was suggested by results from Test 1. Specimen weight gains during this test were incompatible with the weight losses measured after chemically stripping the corrosion product at the end of the experiment unless a magnesium silicate formed part of the corrosion product.

Energy dispersive X-ray analysis (EDAX) showed silicon on specimens from Test 1, and subsequently both EDAX and α -particle back-scattering analysis² proved that some samples from high temperature experiments contained silicon in their corrosion products.

An infrared reflectance spectrum was readily obtained with the specimen from Test 3 (sample *b*) which had a thin (1.8 μ m) transparent film on a smooth metal surface. This spectrum was compared with one recorded for a corrosion product formed in silicate-free solution—sample *a* (specimen 4 of ref. 2). Figure 1 shows that absorption bands due to bound water ($\sim 3,250$ cm⁻¹) and to silicate (at $\sim 1,010$ and 650 cm⁻¹) are present in addition to those of magnesium hydroxide. From the wavelengths of these bands it is inferred that a polymeric hydrated silicate had been incorporated into the corrosion film.

The corrosion film on the specimen from Test 5, being 30 times thicker, probably contained much more silicate than the product on sample *b*, but a satisfactory infrared spectrum could not be obtained from it as very little incident radiation was reflected from the rough surface. An X-ray powder diffraction spectrum of the corrosion product from this specimen showed the presence of both magnesium hydroxide and a magnesium aluminium silicate of the chlorite type, rather than a simple magnesium silicate.

These results demonstrate that silicate contamination can enhance the corrosion of Magnox AL 80 in sodium hydroxide solutions above 60 °C. Even at this temperature gravimetric data suggest participation of silicate in the process, and scanning electron microscopy–EDAX indicates

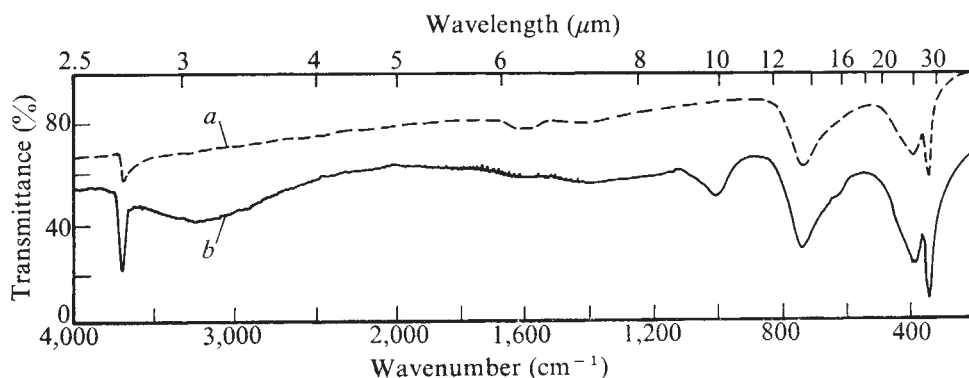


Fig. 1 Infrared reflectance spectra of Magnox specimens coated with thin films of *a*, magnesium hydroxide; *b*, magnesium hydroxide incorporating silicon (Perkin-Elmer 577 grating spectrometer; attenuated air reference).

the preferential formation of a magnesium silicate at grain boundaries. The X-ray diffraction evidence implies that the aluminium content of the alloy, although small, influences the chemical products formed in the corrosion reaction.

It has not been possible here to report on the effect of solution composition on the variation of corrosion rates with time which we have observed, both in experiments involving continuous measurement of gas evolution and by electrochemical techniques. Detailed study of the complex kinetics of the reaction of Magnox with water, its enhancement by silicate and inhibition by fluoride is continuing in our laboratory, and will be reported in due course.

We thank Dr G. C. Allen for communicating his detection of a magnesium silicate by infrared spectroscopy, Dr E. Metcalfe for X-ray analysis of corrosion products and Dr D. A. Hilton for helpful comments. This work is published by permission of the Central Electricity Generating Board.

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Thermoluminescence from γ -ray irradiated normal paraffins

IN recent years, an increasing number of papers have described the trapping mechanisms in organic materials including polymers evaluated by thermoluminescence measurement. Several authors have attempted to correlate the phenomena of thermoluminescence glow peaks and the molecular motions in polymers^{1,2}. Such studies give useful information on transitional motions which may be responsible for thermal glow from the irradiated organic compounds³. This letter presents some of the results obtained from a study of thermoluminescence in normal paraffins above room temperature.

The samples used were commercial normal paraffins in the range *n*-eicosane (C_{20}) to *n*-nonacosane (C_{29}) which were purified by recrystallisation from acetone and/or ethanol hot solution several times. The actual purities estimated from the melting curves were 99.6% for C_{24} and above 99% for the others. All samples were warmed from room temperature to above their melting temperature after irradiation by 0.5 Mrad γ -ray doses, obtained from ^{60}Co at dry ice temperature in air. The luminescence detection apparatus consisted of a photomultiplier (HTV R292) contained in a light-tight box. The temperature was measured by copper-constantan thermocouples attached to the surface of the sample. The calorimetric measurements were made using a Parkin Elmer DSC II type calorimeter. A scanning speed of $5^{\circ}C\ min^{-1}$ was employed.

Figure 1 illustrates a typical thermoluminescence glow curve obtained from irradiated C_{24} and C_{28} . Samples were kept in the dark at room temperature after irradiation and

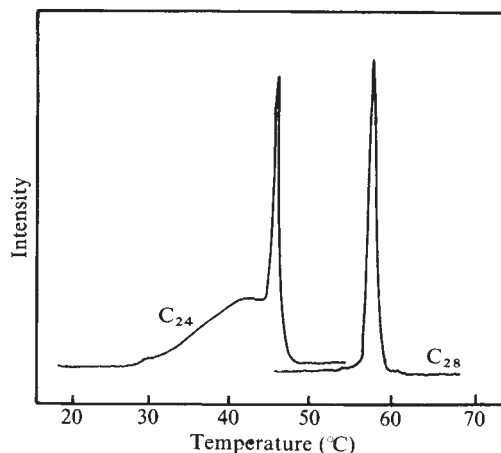


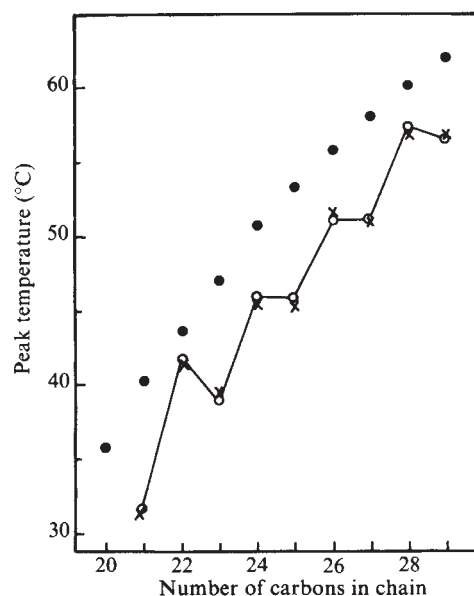
Fig. 1 Thermoluminescence glow curves of irradiated C_{24} and C_{28} . A heating rate of $5^{\circ}C\ min^{-1}$ was used.

during warming. When a sample had given a glow curve, no further light was emitted if it was immediately cooled and reheated. Sharp peaks having very narrow half-line widths of around $0.5^{\circ}C$ were observed in the curves of all the samples, some of which have another broad emission peak on the lower temperature side as shown in Fig. 1. These broad peaks decay relatively fast, while the sharp ones were stable and remained constant in spite of varying with warming rate ranging from 1 to $20^{\circ}C\ min^{-1}$.

It has been clearly demonstrated that a large number of longer paraffins exhibit first order solid state transitions (see Broadhurst⁴). In general, calorimetric measurements are effective in analysing thermodynamic phase transitions. The glow peaks and DSC peaks obtained are plotted against carbon numbers in Fig. 2. It should be noted here that the glow peaks for all samples were in good agreement with solid state transition points. No significant emission was observed at melting. From these results, thermoluminescence at the transition point is not given by simple Boltzman-type variation with temperature.

Equimolar melts of two paraffins with different carbon numbers were also examined. The DSC curve of such a

Fig. 2 Plot of glow and DSC peak temperature as a function of number of carbons in chain. $\times$, glow peak; $\bullet$, melting point in DSC; $\circ$, transition point in DSC.



mixture has two peaks; one was assigned to the melting and the other, of greater temperature band width was assigned to the solid-state transition in the order of descending temperature. Comparing DSC spectra with thermoluminescence peaks, it is also apparent that for binary mixtures, light emission occurs at the solid-state transition point.

The authors wish to thank Mr M. Ikeda and Dr T. Hatakeyama for calorimetric analyses.

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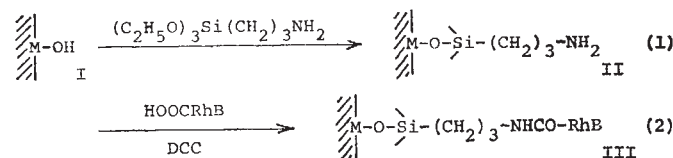
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Photocell using covalently-bound dyes on semiconductor surfaces

ONLY TiO_2 ,¹ SnO_2 ,² and SrTiO_3 ,^{3,4} which do not undergo decomposition upon irradiation when used as photoelectrodes in aqueous media, are stable enough to be used as practical solar energy conversion and hydrogen production devices. The band gaps of these *n*-type semiconductors correspond to the energy of photons in the ultraviolet region. Solar energy reaching the Earth's surface has, however, a spectrum distribution in the longer wavelength range and cannot, therefore, be used effectively by these semiconductors^{5,6}. Spectral sensitisation would solve this problem by extending the sensitivity of the electrochemical photocells to longer wavelengths. In applying this technique to a real photocell another problem may arise in that most of the solar energy is absorbed by the dye molecules in the solution rather than by the dye molecules adsorbed at the electrode-solution interface, though only the excited adsorbed dye molecules can contribute to sensitisation⁷⁻⁹. To cope with this we have developed a new type of photocell¹⁰. In this, the chemically modified SnO_2 or TiO_2 electrode, with rhodamine B as a sensitizer, was in contact with the transparent electrolyte solution containing a reducing agent as a supersensitizer. In this communication, further developments of this subject are described, especially, the correlation between the efficiency of sensitisation and the structure of the modified layer, which gives us the criteria for more successful chemical modifications of electrode surfaces.

In the previous work, the following surface modification procedure was used to synthesise an amide-linked rhodamine B electrode.



M = Sn or Ti, DCC = dicyclohexylcarbodiimide,

HOOCRhB = rhodamine B

The sensitised photocurrent observed at the amide-linked electrode was less than that observed at the conventional untreated electrode in contact with the dye solution.

Since then, we have tried to increase the surface coverage of the amide-linked rhodamine B (III) and ultimately to increase the sensitised photocurrent by improving yields of the silanisation reaction (I→II). It was found, however, that the observed sensitised photocurrent on the modified electrode (III) decreased, against our expectation, with an increase in the surface coverage of aminopropyl derivative (II)¹². This led us to assume that rhodamine B covalently bound via amide bond (III) cannot contribute to the spectral sensitisation, and the observed photocurrent on the amide-linked electrode prepared previously can be attributed to rhodamine B directly bound on the surface via ester bond (IV) with the surface hydroxyl group (I) which remains unreacted with γ -aminopropyltriethoxysilane.

Consequently, the following dehydrative coupling of a carboxyl group of rhodamine B with a surface hydroxyl group of the untreated electrode was tried using dicyclohexylcarbodiimide (DCC) as a dehydrating agent¹¹.



As expected, a sensitised photocurrent spectrum was obtained with the ester-linked rhodamine B electrode and was exactly the same as that observed previously on the amide-linked electrode as shown in Fig. 1. Furthermore, the sensitised photocurrent is about two orders of magnitude

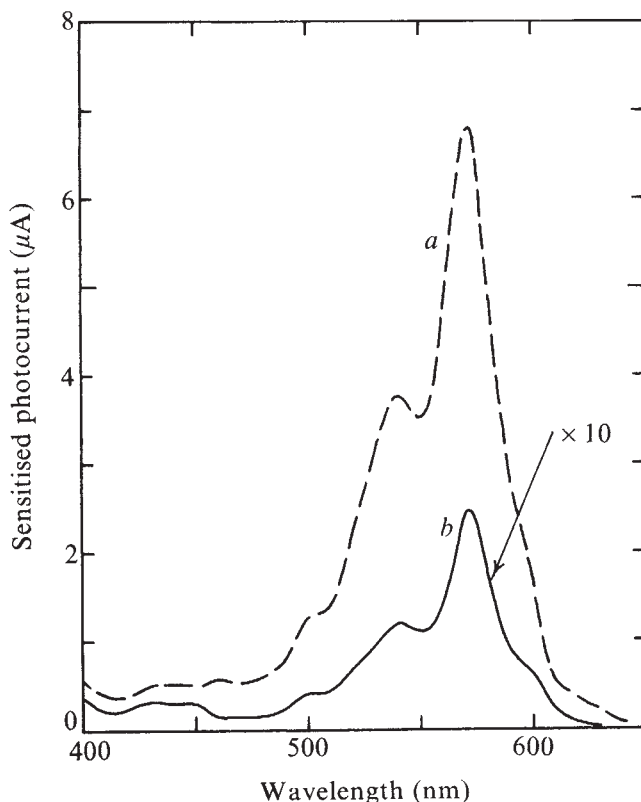


Fig. 1 Spectral dependence of the photocurrent at the chemically modified SnO_2 electrodes with rhodamine B. The cell design used for the photoelectrochemical studies was the same as in the previous paper¹⁰. The optically transparent electrode was polarised anodically at +0.4 V against a saturated calomel electrode (SCE) and illuminated through the electrode (as shown in Fig. 1 of ref. 10). *a*, Photocurrent spectrum sensitised by a sub-monolayer of ester-linked rhodamine B in contact with a Britton-Robinson buffer ($\text{pH} = 3.9$) containing hydroquinone (10^{-2} M) as a supersensitizer. *b*, Photocurrent spectrum observed previously at the amide-linked rhodamine B electrode in contact with aqueous 0.2 M Na_2SO_4 containing hydroquinone (5×10^{-3} M), which is magnified 10 times for comparison.

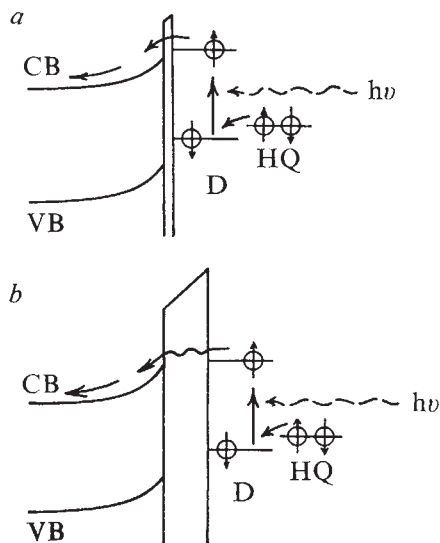


Fig. 2 Schematic presentation of supersensitisation at semiconductor-covalently-bound dye-reductant solution interfaces under anodic bias: D, dye molecule covalently bound on a semiconductor as a sensitizer; HQ, hydroquinone in solution as a supersensitizer; CB, conduction band; VB, valence band. *a*, Ester-linked dye electrode where π electronic system of dye is close to the semiconductor surface. *b*, Amide-linked dye electrode via amino-alkylsilane where π electronic system of dye is apart from the semiconductor surface and a non-conducting alkyl chain intervenes between semiconductor and dye.

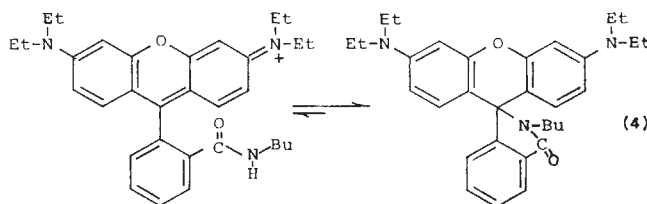
greater on the ester-linked electrode than the photocurrent observed previously on the amide-linked electrode.

The difference in distance of the conjugated π electronic system of rhodamine B from the electrode surface probably accounts for the marked difference in efficiency of the electron injection from excited dye molecules to the conduction band of semiconductor for these ester- and amide-linked electrodes, as shown in Fig. 2. (As these surfaces are chemically predictive, we can evaluate more correctly the height and width of the energy barriers from the properties of binding species such as electron affinity and length of alkyl chains compared with those of the vapour-deposited insulator film such as metal oxides.) In this picture, intervention of the non-conducting alkyl chain in the amide-linked electrode is described as an energy barrier which inhibits the electron injection. This explanation can be supported by the observed difference in electrochemical reactivity of chemisorbed alkenyl hydroquinone on platinum having various lengths of intervening alkyl chain¹³ and by the following observation.

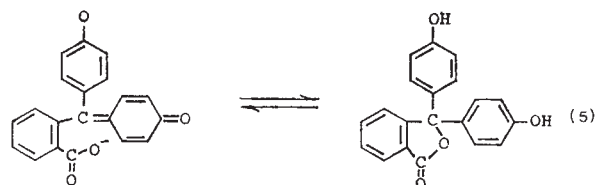
If a monolayer of non-conducting compound at the electrode-solution interface acts as the energy barrier, the electron injection from the dye molecules adsorbed physically at the electrode-dye solution interface should be also inhibited by the presence of such layers. In Fig. 3, a sensitised photocurrent spectrum observed at the aminopropyl modified electrode (II) in contact with the rhodamine B solution is compared with that obtained with the unmodified electrode (I). A pronounced lowering in the sensitised photocurrent was observed by the introduction of nonconducting layers on electrode surfaces.

Parallel to the above experiments, which elucidate why the amide-linked rhodamine B cannot contribute to the spectral sensitisation, the conventional synthesis in solution of the amide and ester derivatives of rhodamine B with butylamine and ethanol was carried out to check the variation of their absorption spectra by derivation. These derivatives were obtained in satisfactory yields by using DCC under the same experimental conditions as used for the surface synthesis. The absorption spectrum of aqueous solution of the ethyl ester is very similar to that of

rhodamine B, whereas the butyl amide is surprisingly almost colourless in most solvents. This is because the amide derivative is stable in the lactam form rather than the amide form as shown in equation (4).



Thus, the deep red colour arising from the quinoid form in the xanthane structure of rhodamine B disappears due to lactam formation of the amide derivative, seen similarly in the acid-base equilibrium of phenolphthalein.



Because the amide-linked rhodamine B loses colour, an insulator model is unnecessary in this special case to explain the lowering of sensitisation efficiency of amide-linked rhodamine B. It is very important, however, to clarify how the intervening non-conducting layer affects the spectral sensitisation process in view of an important role of lipid membranes in photo-oxidation of water^{14,15}. Probably the chemical modification approach as illustrated in this paper is the best method at present to tackle the problem.

In conclusion, if the chemical modification of electrode

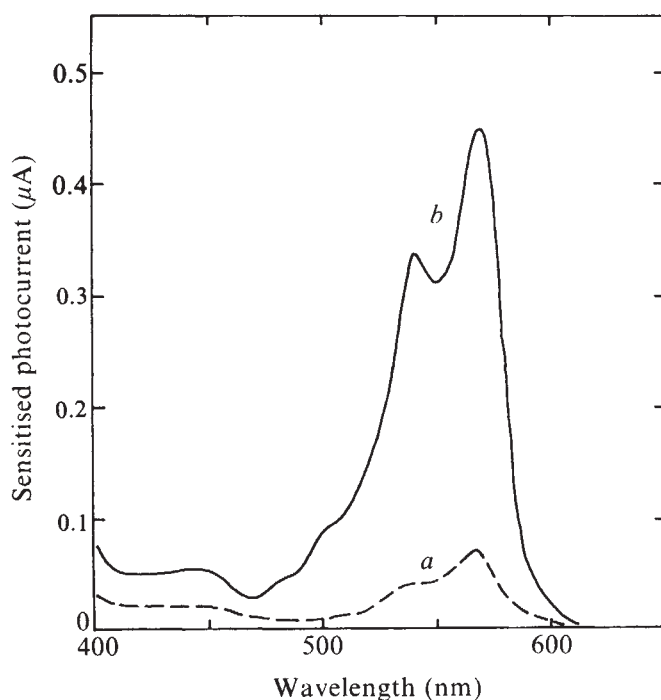


Fig. 3 Spectral dependence of the photocurrent at the electrode-dye solution interface. The conditions for photoelectrochemical measurements were similar to those described in Fig. 1. *a*, Photocurrent spectrum observed at the aminopropyl modified SnO_2 electrode in contact with a rhodamine B solution (5×10^{-5} M rhodamine B + 5×10^{-3} M hydroquinone in 0.2 M Na_2SO_4). *b*, Photocurrent spectrum at the conventional unmodified SnO_2 electrode in contact with the same solution as described in *a*.

surfaces are applied to the solar cells, the sensitisers should be bound as close to the electrode surfaces as possible in order to increase the efficiency of spectral sensitisation. Care must be taken as to how the optical properties of sensitisers are changed by the modification reactions.

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Spanish rock art depicting honey gathering during the Mesolithic

THE end of the Upper Palaeolithic and the subsequent Mesolithic of western Europe coincided with a general change of climate, flora and fauna. Throughout this time the nomadic hunters and food gatherers continued their former activities and perfected their techniques in a changing environment, before the introduction of agriculture and the change to a sedentary way of life. In Spain, this period is associated with a type of rock art known as 'art of the Spanish Levant', because it occurs only within a limited area of the mountainous coast of eastern Spain; it is provisionally dated approximately from 8,000 to 2,000 BC. Its outstanding features are the appearance of motion and the fact that the central figures are mainly human, around which animal figures are grouped. We report here a scene we discovered last year at one of the most important Levantine rock-art zones, the Barranco de la Valltorta (Castellón), which is a dried out, deep and eroded river bed. It shows wild honey gathering activities and depicts the first definite ladder constructed of side ropes, with visibly rigid intersecting rungs, which are straight and even, and which must be sticks prepared for this purpose.

A stretch of about 7 km of the Valltorta contains 14 rock-art sites, totalling 39 painted rock shelters, located on both banks of the ravine (Fig. 1); these were all discovered in 1917. The paintings of the left bank have been recorded in part and published^{1,2,5}, while those of the right bank, located on steep cliffs and very difficult to reach, were reputed to contain only meaningless remains of paintings. During a study of the origins and development of Levantine rock art, we have checked and redrawn all known sites. On the right bank we found some paintings which could still be interpreted and correlated because the only damage they had sustained was due to frost and natural erosion. Difficulty of access had protected them from the moistening and rubbing which has defaced so many Levantine rock shelters.

The scene we are reporting is inside rock shelter no. 4,

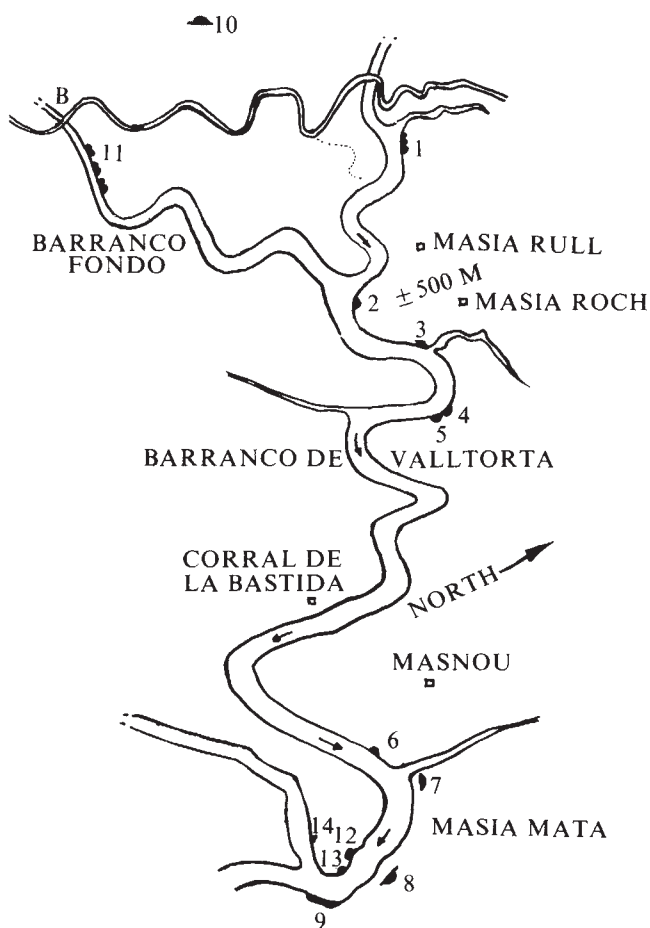


Fig. 1 The Barranco de la Valltorta and its 14 Levantine rock art sites: 1, El Civil; 2, Cueva dels Tolls Alts; 3, Cueva Rull; 4, Els Caballs; 5, Cueva del Arco; 6, Mas d'en Josep; 7, Cueva Alta del Llidoné; 8, Calsas del Matà; 9, La Saltadora; 10, Coveta de Montegordo; 11, Cingle de la Eremita del Barranc Fondo; 12, Cingle dels Tolls del Puntal; 13, Cueva Grande del Puntal; 14, Covetes del Puntal.

counting from the left, of the Cingle de la Eremita del Barranc Fondo, which is a small subsidiary of the Valltorta. This group of six rock shelters, of total length 96 m, is on the left bank of the narrow Barranc Fondo and can be reached only from the upper plateau. Rock shelter No. 4 contains several paintings in various shades of red, from a faded pinkish red to a reddish Sienna brown. In general the brownish paintings are the oldest. The panel is on the right side of the rear wall and undoubtedly depicts wild honey gathering activities (Fig. 2).

Human figures climbing ropes or ladders can be seen in several Levantine sites, but cannot always be interpreted as evidence for honey gathering. One well known scene is that of La Araña (Valencia) which has been reproduced many times³; a lesser known group is that of Dos Aguas (Valencia)⁴, and we believe a third to be visible at Los Trepadores (Teruel)⁶. The new honey gathering scene would thus be the fourth.

The rock surface of the rear wall is uneven and shows many small fissures, crevices, and cup-like holes. The faded red colour of the painting indicates a late phase of Levantine art; this is confirmed by the small size of the human figures, as the tendency is for figures to become smaller and more schematic before the disappearance of this type of rock art. Several small cup-like holes at the top were the starting-point of the composition, for they depict the wild bees' nest. The similar scenes of La Araña and Dos Aguas also have a natural hole as a starting point. The total height of the scene, starting from the holes, is 52 cm.

Below the holes, two fissures have been made more conspicuous by outlining with red paint; at the right, the painted lines converge to a central stem evocative of the branch of a tree; on the left, the faded outline of a small animal, which may be a wild goat, can still be traced. Both fissures converge into the rough shape of a V; at this point, there is a squarish object with some appendages radiating out from the corners. This may represent a leather bag or basket for the gathering of honey. The actual ladder begins here and continues downwards for about 40 cm. The bees surrounding the ladder are disproportionately large, but this is also the case in the other honey gathering scenes.

The ladder is of complex construction, being the first of this type to be observed in Levantine rock art; it is a much more sophisticated version of the plain ropes attached to forked sticks at La Araña. At some places, sticks are attached to the side ropes, as can be seen on the segment of ladder trailing on the ground at lower left; at other places, the rope coils in a serpentine pattern. The thickening of the rungs at the point of contact with the ropes may indicate a knot, and the thickening of the side ropes may be a doubling or tripling of the rope at that particular point.

On the ladder, there are five human figures; the first two, at the top, are climbing in a doubled-up posture, grasping the

Fig. 2 The honey gathering scene from rock shelter no. 4 of the Cingle de la Ermita del Barranc Fondo.



Fig. 3 The honey gathering scenes already known in Levantine rock art: a, La Araña; b, Dos Aguas; c, Los Trepadores.

ladder with both hands, and feet; the second figure from the top may be bearded. The third figure from the top seems to be jumping off or falling down; a fourth figure is again climbing up and the fifth, at bottom, is definitely jumping down from the ladder. A vertical fissure runs more or less parallel to the painted ladder.

At the bottom, there are 12 very small human figures, mainly female, a small animal and other remains; two male figures may have bows. The female figures do not seem to be engaged in any particular activity and have no ritual or ceremonial meaning, such as raised arms or dancing postures. This part of the scene is badly damaged by erosion and the small size of the figures (3–5 cm) does not make it easy to connect them with a honey-gathering activity, unless they are supposed to represent onlookers. This scene shows a much more advanced technology than those known from the same periods (Fig. 3), at La Araña (Valencia), Dos Aguas (Valencia) and Los Trepadores (Teruel), and it therefore throws new light on honey-gathering activities during the late Ice Age. Although the use of ropes is depicted in the other scenes, it is not known what type of fibres were involved. But the newly discovered scene shows a ladder built of ropes and wooden rungs, such as are still in use, mainly for mountaineering. The whole construction must have been very strong, as it supports the weight of five people (roughly 300–350 kg); this implies the use of adequately thick and straight sticks of similar weight for the rungs, and firmly tied knots to keep them in place. It would be interesting to know how the top of the whole contraption was attached: it was probably knotted around a projecting rock surface, near the rock fissure containing the wild bees' nest. It is not possible to speculate on the total length of the ladder, for the human figures are probably not drawn to scale.

Honey-gathering scenes are frequently depicted in South African rock art; in one example, at Anchor Shelter, Natal Drakensberg, the ladders are remarkably similar to that shown in the new scene^{7,8}. In European rock art, however, the four

scenes discussed here are the only indications of this type of activity among Late Stone Age hunters and food gatherers.

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The ornithopod dinosaur *Dryosaurus* and a Laurasia–Gondwanaland connection in the Upper Jurassic

COMPARISONS of Upper Jurassic ornithopod dinosaurs show that *Dysalotosaurus lettow-vorbecki* (Tendaguru Beds, Tanzania, East Africa) is referable to the genus *Dryosaurus* (Morrison Formation, western North America). This extended distribution of *Dryosaurus* provides additional evidence for a land connection between Laurasia and Gondwanaland sometime in the Upper Jurassic.

The close similarities of the Upper Jurassic dinosaur faunas of the Morrison Formation of the western United States and the Tendaguru Beds of Tanzania, East Africa (Tables 1, 2) are often cited as evidence for the presence of a Jurassic land connection between Laurasia and Gondwanaland (Fig. 1a). As regards genera common to both faunas (Table 1), most

Table 1 Upper Jurassic dinosaur faunas from Morrison Formation of North America and Tendaguru Beds of East Africa

Saurischia: Theropoda	Morrison	Tendaguru
Coeluridae	<i>Coelurus</i> , <i>Ornitholestes</i>	<i>Elaphrosaurus</i>
Megalosauridae	<i>Ceratopsus nasicornis</i> new genus*	<i>C. (?) roechlingi</i>
		<i>Megalosaurus (?)</i>
Allosauridae	<i>Allosaurus fragilis</i>	<i>A. (?) tendagurensis</i>
Incertae sedis	<i>Iliosuchus</i> ²⁰ , <i>Marshosaurus</i> ³⁰	1 or 2 small species ¹¹
Sauropoda†		
Cetiosauridae	<i>Haplacanthosaurus</i>	
Brachiosauridae	<i>Brachiosaurus altithorax</i>	<i>B. brancai</i>
Camarosauridae	<i>Camarosaurus</i>	
Apatosauridae	<i>Apatosaurus</i>	
Diplodocidae	<i>Barosaurus lentus</i> <i>Diplodocus</i>	<i>B. africanus</i>
Dicraeosauridae		<i>Dicraeosaurus</i>
Titanosauridae		<i>Torniera</i>
Ornithischia: Ornithopoda		
Fabrosauridae	<i>Nanosaurus</i>	
Hypsilophodontidae	<i>Dryosaurus altus</i> <i>Othnielia</i> † n. g. <i>Camptosaurus</i>	<i>D. lettow-vorbecki</i>
Iguanodontidae		
Stegosauria		
Stegosauridae	<i>Stegosaurus</i>	<i>Kentrosaurus</i>

Species given only when genus present in both faunas.

* To be described by author and J. A. Jensen of Brigham Young University, Provo, Utah.

† Families follow Janensch⁷, valid Morrison genera following McIntosh and Berman³¹.

‡ *Othnielia* n. g., named after Othniel Charles Marsh, type species *Nanosaurus rex* Marsh³² (see ref. 13); type species of *Laosaurus* Marsh³³, *L. celer* Marsh³³, is a *nomen nudum* with *L. gracilis* Marsh³³ and *L. consors* Marsh³⁴ referable to *Othnielia rex* (Marsh).

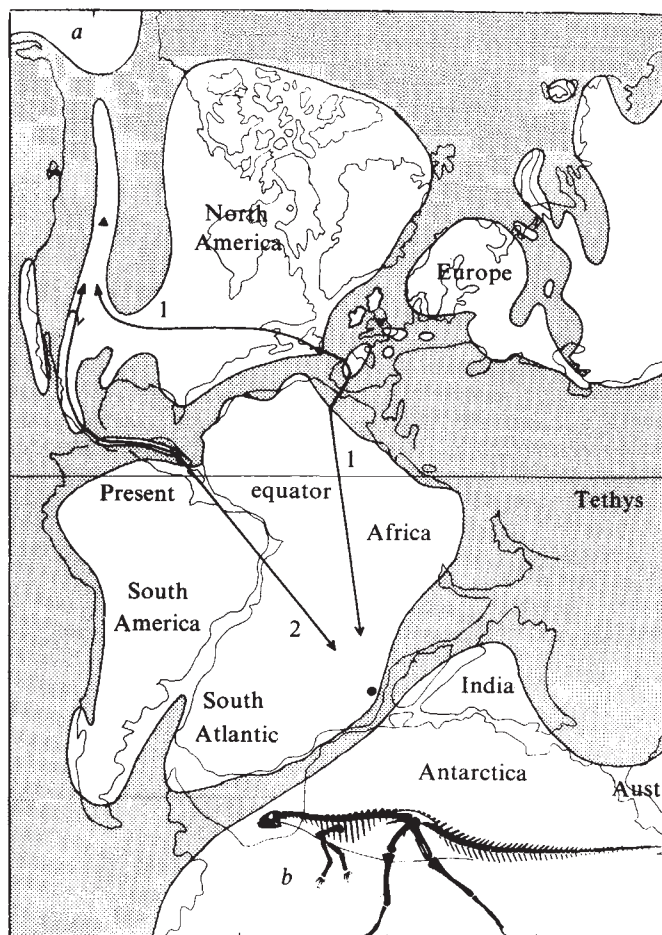


Fig. 1 a, Location and distribution of land in the western part of Laurasia and Gondwanaland during the Upper Jurassic (Kimmeridgian), after Ager²⁴; —, reconstructed coast lines; —, present coast lines; ▲, *Dryosaurus altus* localities, western USA; ●, *Dryosaurus lettow-vorbecki* locality, Tanzania, East Africa; ▼, *Dryosaurus* sp. locality, England; Aust., Australia; 1, possible dispersal route via western Europe; 2, possible dispersal route via Central and South America. b, Skeletal reconstruction of *Dryosaurus lettow-vorbecki*, complete length of animal about 2.5 m, based on mounted skeleton HMN dy I¹⁴.

authors cite the enormous sauropod *Brachiosaurus*^{1–5}, and some also mention the smaller sauropod *Barosaurus*^{1,2,6,7}. The complete anatomy of the Tendaguru species of *Brachiosaurus* is well known^{7,8}, but the material of *Barosaurus* is less complete⁷. The referral of the Tendaguru species to these genera is probably correct but as the type species of *Brachiosaurus* and *Barosaurus* from the Morrison Formation is so far each represented by only one partial skeleton described^{9,10}, the possibility that one or both of the Tendaguru species represent separate but related genera cannot be completely eliminated. The large carnivorous theropods *Allosaurus* and *Ceratopsus* are represented by complete skeletons from the Morrison Formation, but the Tendaguru material tentatively referred¹¹ to these genera is extremely fragmentary. The one good skeleton of a medium sized theropod (*Elaphrosaurus*)¹¹ from the Tendaguru Beds is clearly distinct from the Morrison coelurids *Coelurus* and *Ornitholestes*. The stegosaurs or plated dinosaurs are well represented in both faunas, but *Stegosaurus* and *Kentrosaurus* are distinct and not closely related. The bipedal ornithopods are represented by four taxa from the Morrison Formation: the small fabrosaurid *Nanosaurus agilis*¹²; the hypsilophodontids *Othnielia rex*¹³ (Table 1), and *Dryosaurus altus*; and the medium sized iguanodontid *Camptosaurus dispar*. Comparisons of the good material of *Dryosaurus altus* with the excellent material of *Dysalotosaurus lettow-vorbecki* (Fig. 2), the only Tendaguru ornithopod described¹⁴, show that these taxa are congeneric as *Dryosaurus altus* (Marsh) and *Dryosaurus*

lettow-vorbecki (Pompeckj). The osteology of these species of *Dryosaurus* is clearly distinct from that of the other hypsilophodontids *Othnielia*¹³, *Hypsilophodon*¹⁵ and *Parksosaurus*.

The skulls (Fig. 2a-d) show a few differences, but some of these (posterior region Fig. 2a and c, width Fig. 2b and d) result from crushing of the Morrison skull. In addition, two skulls¹⁵ of *Hypsilophodon foxii* show greater differences in sutural pattern anteroventral to the orbit than do those of

Dryosaurus. The main distinguishing character in the skull of *Dryosaurus altus* (Fig. 2a, b) is the more elongate supraorbital (Fig. 2a). The humeri (Fig. 2i-l) have low deltopectoral crests and low condyles distally (Fig. 2i, k, l). In the pelvic girdle (Fig. 2m-r), the main body of the ilium is low with an obliquely truncated posterior end, and the brevis shelf is broad; the distal half of the ischium is curved and bar-shaped with a proximally placed obturator process; and the anterior process of the pubis

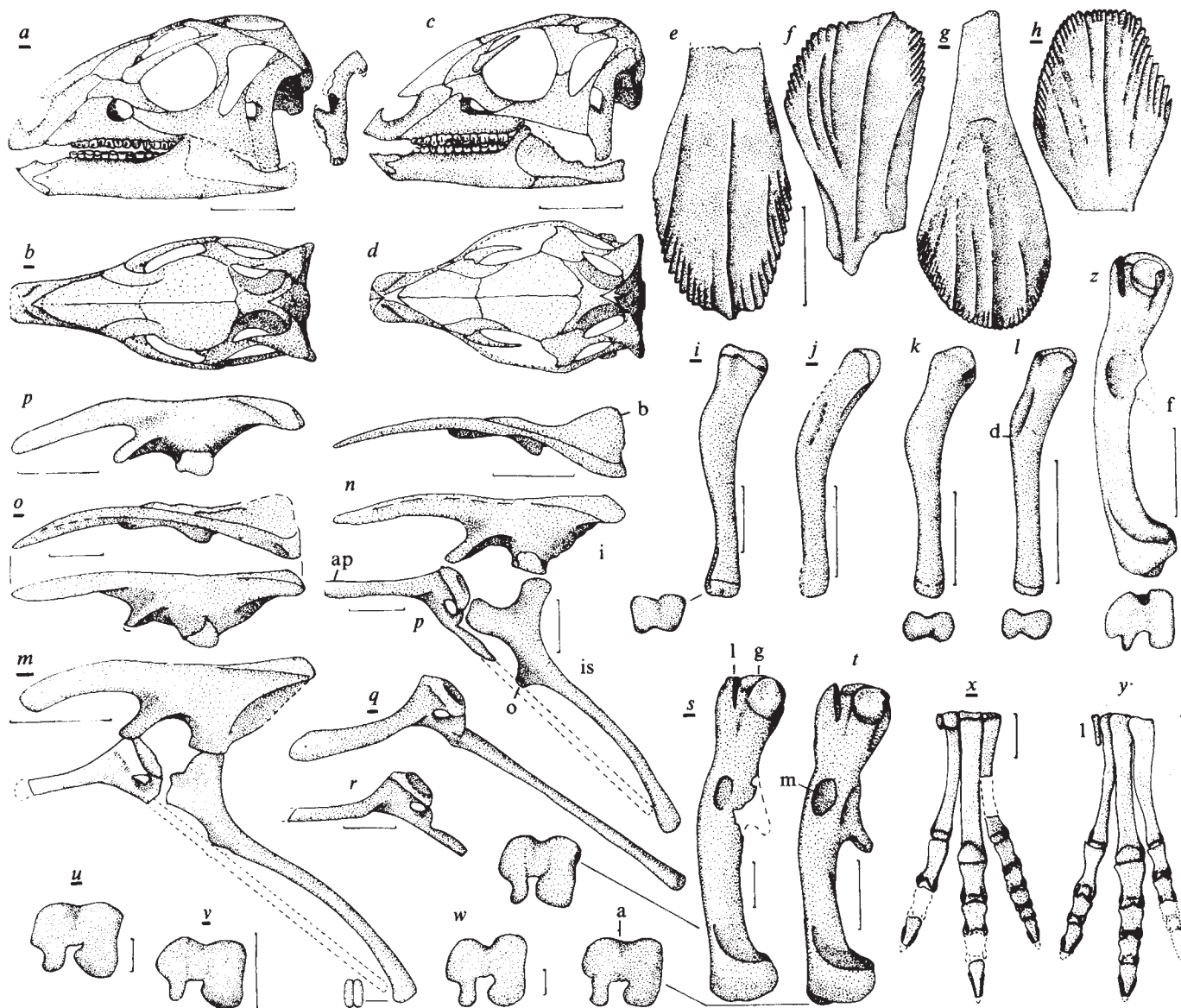


Fig. 2 Comparative anatomy of hypsilophodontid dinosaurs *Dryosaurus altus* from Morrison Formation of western USA (a, b, g-j, m, o, q, s, u, v, x; identifying letters underlined to facilitate comparisons); *Dryosaurus lettow-vorbecki* from Tendaguru Beds of East Africa (c-f, k, l, n, p, r, t, w, y); and holotype of *Dryosaurus ? canaliculatus* Galton¹⁶ (type species of *Valdosaurus* n. g.) from Wealden of England (z) in lateral view (a, c, e, g, i-r), medial view (f, h, s, t, z), dorsal view (b, d, n, o, x, y) and/or distal ends (i-l, s-w, z); all bones drawn as if from left side except medial views with distal views that are from right side. a, skull of CM 3392 with quadrate YPM 1915; b, skull CM 3392; c, skull HMN dyA, modified after Janensch¹⁴; d, skull HMN dyA, modified after Janensch¹⁴; e, slightly worn maxillary tooth after Janensch¹⁴; f, moderately worn dentary tooth after Janensch¹⁴; g, unworn maxillary tooth YPM 1915; h, unworn dentary tooth YPM 1915; i, humerus YPM 1915; j, humerus AMNH 834; k, humerus UT 1495/22; l, humerus UT 1495/23; m, pelvic girdle AMNH 834; n, reconstruction of pelvic girdle (HMN dyI, dyII, dy35) with dorsal view of ilium (HMN dyII); o, ilium DNM 1016 in dorsal and lateral views; p, ilium UT 1495/9; q, pubis YPM 1884; r, pubis UT 1495/6; s, femur YPM 1915; t, femur HMN dy36; u, femur CM 21786; v, femur CM 21786; w, femur UT 1495/14; x, pes CM 21786; y, pes HMN dyV; z, femur BMNH R185, after Galton¹⁶. a, Anterior intercondylar groove; ap, anterior process of pubis; b, brevis shelf; d, deltopectoral crest; f, fourth trochanter; g, greater trochanter; i, ilium; is, ischium; l, lesser trochanter; m, depression for *M. caudi-femoralis longus*; o, obturator process; p, pubis; l, vestigial first metatarsal. Scale lines represent 5 cm (a-d, i-z) or 1 cm (e-h). YPM 1915 is holotype of *Laosaurus altus* Marsh, the type species of *Dryosaurus* Marsh³⁴. Specimens shown in j, m, o, u, v and x will be fully described elsewhere³⁵. I thank the people indicated for their help while studying the figured specimens in their respective institutions indicated by the following abbreviations: AMNH, American Museum of Natural History, New York (E. S. Gaffney); BMNH, British Museum (Natural History), London (A. J. Charig, C. A. Walker); CM, Carnegie Museum, Pittsburgh (D. S. Berman); DNM, Dinosaur National Monument, Jensen, Utah (T. E. White, J. Adams); HMN, Humboldt Museum für Naturkunde, East Berlin (W.-D. Heinrich, J. Helms, H. Jaeger); UT Universität der Tübingen (F. Westphal); YPM, Peabody Museum Yale University, New Haven (J. H. Ostrom).

is slender with a variably developed ventrolateral edge. The femora (Fig. 2s-w) possess the same suite of characters: a deep cleft separating the lesser and greater trochanters; the deep depression level with the proximally placed fourth trochanter is set well anteriorly on the shaft; distally the medial edges are squared off, there is an anterior intercondyle groove, and the posterior lateral condyle is thin. In the pes the first metatarsal is rudimentary so there were only three movable toes (Fig. 2x and y).

Fragmentary evidence indicates the presence of *Dryosaurus* in Europe. A slender tibia (Sedgewick Museum, University of Cambridge specimen number J46889) from the lower Oxford Clay (Middle Jurassic, Middle Callovian) of Fletton near Peterborough, Northamptonshire (Fig. 1a) is probably referable to *Dryosaurus*. Femora (Fig. 2z) from the Wealden (Lower Cretaceous) of southern England that were tentatively referred¹⁶ to *Dryosaurus* differ from femora of that genus in having a deeper anterior intercondylar groove, the antero-medial margin of which is squared off. A new genus *Valdosaurus* (see Fig. 2z) is erected for this material, which probably represents a Cretaceous descendant of *Dryosaurus*.

The presence of the hypsilophodontid dinosaur *Dryosaurus* in the Upper Jurassic (Upper Kimmeridgian, base of Tithonian)^{1, 17} of western United States and Tanzania provides important additional evidence for the presence of a land connection between Laurasia and Gondwanaland (Fig. 1) sometime in the Upper Jurassic (Kimmeridgian or possibly Oxfordian). A North America-Europe-Africa route is usually assumed,

Table 2 Comparisons between Morrison and Tendaguru dinosaur faunas

	Genera	Families
Morrison fauna (N or N_2)	18	14
Tendaguru fauna (N or N_1)	11	9
Morrison endemics (E)	9	0
Tendaguru endemics (E)	5	1
Taxa in common (C)	5	7
Simpson's coefficient ($C/N_1 \times 100$)	45%	77%
Coefficient of difference ($(1-C/N_2) \times 100$)	72%	50%
Degree of Morrison endemism ($E/N \times 100$)	50%	0%
Tendaguru endemism	45%	9%

with the connection located in southwest Europe and northern Africa¹⁻⁵ (Fig. 1). Support for this route is provided by the occurrence of *Apatosaurus* and *Brachiosaurus* in the Upper Kimmeridgian of Portugal¹⁸. But, Jurassic deposits in the critical region (southern Spain and Morocco) are largely marine^{1,2,17} and, in addition, it is impossible to restore the coast lines in this area accurately as a result of the complexity of movements of the many small areas involved in the Alpine System orogeny^{4, 19}. If a European route was involved then presumably a land bridge emerged from the shallow sea between Europe and North Africa for a brief period or periods in the Upper Jurassic.

No dinosaurs have yet been reported from the Jurassic deposits of Central America or northern South America⁶, but the possibility that the Morrison-Tendaguru land connection involved the route North America-South America-Africa (Fig. 1) should not be ignored. The southern end of the South Atlantic opened first but a land route was still present between South America and Africa in the Lower Cretaceous (Aptian)²⁰. The Central Atlantic began to open in the early Jurassic^{1,19} with the Azores-Gibraltar ridge marking the line of shear between Europe and Africa as the latter separated from North America¹. Gordon²¹ shows a wide connection between the Pacific and the Central Atlantic throughout the Jurassic but his maps, like those in Colbert⁵, are based on the reconstructions by Dietz and Holden²². In other reconstructions, North and South America are shown much closer together^{1, 22-26} and even in contact²⁷. Berggren and Hollister²³ consider that there was a marine connection for

much of the Jurassic across Central America between the Pacific and the Central Atlantic, but note that this connection may have been intermittent during the Upper Jurassic. On the basis of marine invertebrates, Hallam²⁸ shows that a direct marine connection was not established until after the uppermost Middle Jurassic (Lower Callovian) and Ager²⁴ dates the connection as lower Upper Jurassic (Oxfordian)²⁴. The maximum extent for Jurassic seas was reached during the Oxfordian and Kimmeridgian, when between a quarter and a third of the total continental area was covered¹. While much of western Europe and northern Africa was submerged, there were proportionally shorter stretches of shallow sea separating North and South America^{1,17,24-26} (Fig. 1) or possibly no sea at all²⁷.

The presence of *Dryosaurus* and other similarities (Tables 1, 2) in the Morrison and Tendaguru faunas clearly establishes the availability of a land connection between Laurasia and Gondwanaland sometime in the Upper Jurassic (Oxfordian?, Kimmeridgian) or possibly in the uppermost Middle Jurassic (Upper Callovian). But whether this connection was through Central America or western Europe cannot be determined at the moment.

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Vesicular-arbuscular mycorrhiza in some aquatic vascular plants

THE occurrence of vesicular-arbuscular (VA) mycorrhiza in flowering plants is a common phenomenon. Harley¹ pointed out that it is easier to list the few plants from which the endophyte is normally absent. It has been stated that aquatic plants are not infected by VA mycorrhiza^{1,2}.

Read *et al.*³ found infection levels to be low and often absent in marsh plants and infection in *Cladium* spp. occurred only during dry periods⁴. During an investigation of root hair development in *Littorella uniflora*, *Lobelia dortmanna*, and *Isoetes lacustris* VA mycorrhiza was discovered in connection with evaluation of root uptake of inorganic carbon and nutrients⁵. Other submerged and a few emergent aquatic plants were also investigated.

Plants with intact root systems were collected in January and March 1977 in four softwater, oligotrophic lakes in central Jutland, Denmark. All samples were taken from 0.3–0.8 m depth to make sure that the plants had not at any time been above water level. The isoetid species were examined for development of root hairs by phase-contrast microscopy. The percentage of VA-infection was estimated by the root-slide technique³. Roots of randomly selected plants were cut into 1-cm segments, cleared and stained by the method of Phillips and Hayman⁶. All segments were examined for infection.

An *Endogone* sp. forming VA mycorrhiza was observed in five of the seven species examined (Table 1). Chlamydo-spores, vesicles and arbuscles were present in January

Table 1 Percentage VA-infection in aquatic plants from four oligotrophic lakes, March 1977

Species	Kalgaard		Rævsø		Hampen		Almind	
	%	N	%	N	%	N	%	N
<i>Littorella uniflora</i>	82	44	22	79	36	76	96	92
<i>Lobelia dortmanna</i>	55	69	30	57			46	74
<i>Isoetes lacustris</i>	0	48	0	60	0	36		
<i>Myriophyllum alterniflorum</i>					0	43		
<i>Callitriche hamulata</i>					54	26		
<i>Phragmites australis</i>					+		+	
<i>Eleocharis palustris</i>					15	27		

N, Number of segments examined; +, infection observed.

but only vesicles and arbuscles were found in March. Of the isoetid species frequency of infection was highest in *Littorella uniflora* while *Lobelia dortmanna* was less infected. In agreement with earlier observations¹ no mycorrhiza was found in *Isoetes lacustris*. *Callitriche hamulata* was not infected in January, but intercellular vesicles were present in March. The type of vesicle was different from that observed in the other plant species. *Phragmites communis* and *Eleocharis palustris* were infected but the level of infection was low. At present nothing conclusive can be said about the differences of infection level of the same species in different lakes, but it is known that in terrestrial plants infection is heaviest in infertile soils³.

Development of root hairs by the isoetid species is shown in Table 2. Root hairs were present in *Isoetes lacustris* but not in *Littorella uniflora* and *Lobelia dortmanna*. The

Table 2 Development of root hairs in three isoetid species

Species	Kalgaard	Rævsø	Hampen	Almind
<i>Littorella uniflora</i>	—	—	—	—
<i>Lobelia dortmanna</i>	—	—	—	—
<i>Isoetes lacustris</i>	+	+	+	—

+, Root hair development present; —, development absent.

observation by Shannon⁷ that *Lobelia dortmanna* has root hairs could not be confirmed. The plants investigated by Shannon were seedlings cultivated in water, however, which may have influenced the development. In contrast our plants were mature and growing submerged in sandy sediments.

Ionic absorption and especially uptake of phosphorous is enhanced in plants infected by VA mycorrhizal fungi^{8–10}. As the roots of aquatic vascular plants also function as

organs for ionic absorption² the infection of VA mycorrhiza may have great influence on nutrient uptake, especially in *Littorella uniflora* and *Lobelia dortmanna* that are normally growing in sandy sediments poor in nutrients. The advantage of VA to these plants is further enhanced by the fact that the symbiotic system operates more effectively in low nutrient regimes⁸. The heavy infection of *Littorella uniflora* and *Lobelia dortmanna* is in agreement with the suggestion of Baylis¹¹ that plants with poorly developed root hairs may be obligatory mycotrophs in P-deficient soils. In contrast to *Littorella uniflora* and *Lobelia dortmanna*, *Myriophyllum alterniflorum* has its greatest absorptive surface in the free water and thus is less dependent on the root system which may explain the absence of mycorrhiza in this species.

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Evolution of long-distance alarm calls in Kloss's gibbon

WE present evidence here that proximity of close kin is a plausible hypothesis to explain the evolution of long-distance alarm calls in Kloss's gibbon (*Hylobates klossii*). We discovered two alarm calls produced by adults of this monogamous ape that provide good locational cues and are audible for much greater distances than required to signal the immediate family. Our further finding that at least some Kloss's gibbons settle in territories adjacent to their parental families when they mature and establish their own reproductive groups suggests that the adaptive significance of these calls is to warn neighbouring relatives of the presence and location of danger.

Many species of birds and mammals, including primates, emit vocal alarm signals when a potential predator is detected^{1,2}. Alarm calls of birds in particular have received much attention because they well illustrate the adaptive significance of signal structure³ and because they have been interpreted as animal altruism evolved by kin selection⁴. Interpretation of alarm calls relative to kin selection theory, however, has been impaired by the paucity of data on the distribution of related individuals in wild populations. It is confined further by the lack of knowledge on how predators respond to alarm calls of their prey¹. These difficulties are partially overcome in this study of alarm calls given by Kloss's gibbons.

Kloss's gibbon is strictly endemic to the four Mentawai Islands, 85–145 km off the west coast of Sumatra, Indonesia⁵. This study was conducted in evergreen rain forest on Siberut, the northern-most Mentawai Island, from September 1971 to October 1974, particularly during 387 d at Teitei Peleigei Forest (1°24'S, 99°1'E) where we identified 15 gibbon groups and their territories. Kloss's gibbons live in monogamous families of two to six individuals and defend territories of 7–11 hectares (ref. 5 and R.L.T. in preparation).

Ten kinds of Kloss's gibbon vocalisations have been identified (ref. 6 and Table 1). Two of them, produced by adults of both sexes, are long-distance alarm calls referred to as sirening and alarm trills (Fig. 1). They were audible to us on the ground up

to 800 m away (based on marked, tape-measured trails) and indicate specifically the detection of a potential predator. Audibility range of gibbon calls to a man on the ground may be different from that to another gibbon, up in the forest canopy. It also may vary with time of day⁷ and local atmospheric conditions (K. Henwood and A. Fabrick, in preparation). Nonetheless, our ground-level determinations are useful to illustrate relative audibility of various calls in the Kloss' gibbon repertoire. They show that the only vocalisations with audibility ranges as great as those of sirening and alarm trills are male and female songs (Table 1), which apparently function in maintenance of territorial organisation among neighbouring groups⁸.

We noted 38 bouts of sirening and alarm trills during our study at Tei-tei Peleigei Forest, 21 times from gibbon families disturbed by one of us and 12 times in response to native hunters. Cause in the other five cases was undetermined, although hunters may have been involved. Sirening and alarm trills that we heard during surveys elsewhere in Siberut in 1971 were also in response to human disturbance. Man is the major and perhaps only stimulus eliciting these long-distance alarm calls. This is appropriate since Siberut natives depend heavily on primate flesh for protein, and there are no other predators on Kloss's gibbon except possibly the reticulated python⁵. The hunters, armed only with a palmwood bow and unfletched poisoned arrows, depend on stealth to approach their prey. Despite several hundred years or more of being hunted by Siberut natives, the Kloss's gibbon population is denser than any other hylobatid population known⁵.

The repetitive, frequency-modulated syllables of sirening, the rapidly repeated elements in the alarm trill, and the rich harmonic structures of both (Fig. 1), are characteristics which facilitate directional location of a signaller by recipients making binaural comparisons of phase, time and intensity^{1,3}. Our own experience at distances to 800 m is that sirening, which normally (>80% of instances) begins before alarm trills, sounds the general alarm that a predator is in a specified area. Subsequent alarm trills pinpoint its location, providing more accurate information about direction and distance. Redundancy of the calls provides recipients ample opportunity to obtain this information: during tape-recorded bouts of four alarm-calling gibbon pairs, sirening occurred at an average rate of once every

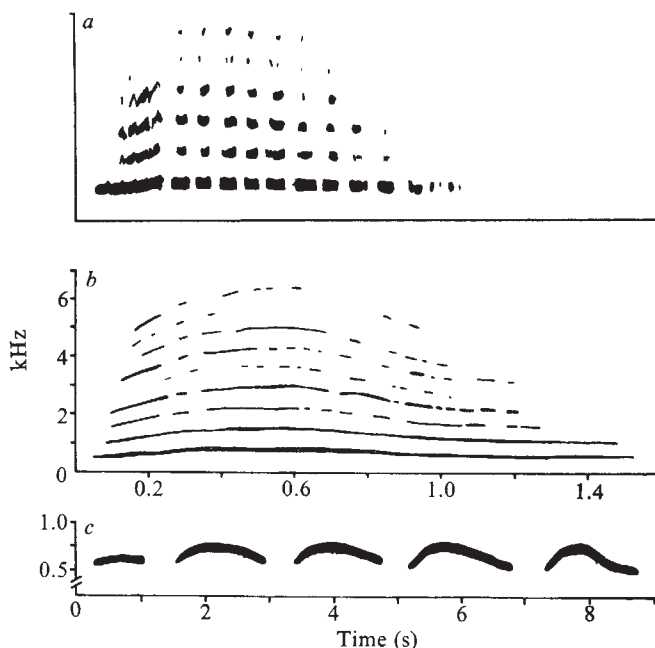


Fig. 1 Ink tracings of audiospectrograms of Kloss's gibbon loud alarm calls. *a*, Alarm trill (time and frequency as in *b*). *b*, One syllable of a sirening call to show harmonic structures. *c*, Example of a complete seven-syllable sirening call (harmonics were deleted in producing the spectrogram, only fundamentals are shown). Mean number of syllables per sirening call is 4.8 (s.d. ± 1.4 , $R = 3-7$, $N = 11$) and mean duration of the call is 7.8 s (s.d. ± 2.4 , $R = 5-12$, $N = 11$). Mean duration of the alarm trill is 1.0 s (s.d. ± 0.1 , $R = 0.93-1.28$, $N = 21$). In 75% (39/52) of cases the alarm trill is preceded by one to three loud 'whups'. Tenaza recorded these vocalisations in the field with a Uher 4400 tape recorder (run at 3 3/4 inches per second) and a Sennheiser 405-S microphone fitted to an aluminium parabola 60 cm in diameter. Audiospectrograms were produced with a Kay Elemetrics sound spectrograph. *a* and *c* were made on wide band setting, *b* on narrow band.

95 s per pair and alarm trills once every 19 s per pair. Thus an average bout of 9.4 min (s.d. ± 7.5 min, $R = 2-27$, $N = 21$) includes about 30 alarm trills and 6 sirening calls.

Aboriginal hunters attempting to approach undetected to within bow and arrow range (50 m or less) of Kloss's gibbons usually abandon the effort if the gibbons discover them, although they normally pursue the other primates (*Presbytis potenziani*, *Simias concolor*, and *Macaca pagensis*) in similar circumstances. Hunters give two reasons for this, first alerted gibbons are unusually difficult targets because of their rapid movement in the upper reaches of the canopy, and second, the gibbons' loud alarm calls alert other primates in the vicinity which lowers the hunter's chances of success. We observed all four species of anthropoid primates in Siberut confronting hunters but only the gibbon produced predator-specific alarm calls audible to such great distances. Although the Mentawai langur (*P. potenziani*) emits an alarm call audible to about 500 m, it is given when confronting conspecifics and gibbons as well as when encountering humans⁹.

Apparently because a Kloss's gibbon does not further endanger itself by revealing its position to the predator (human hunters), signals that not only announce the presence of danger but also inform recipients of its precise location have been allowed to evolve. If hunters were more capable of capturing alerted gibbons, selection might have resulted instead in alarm calls that are difficult to locate, like the ventriloquial whistles certain passerines and rodents emit when a predator such as a flying raptor is detected^{3,10,11}.

Sirening and alarm trills may have evolved simply to warn mates and offspring. But, members of a Kloss's gibbon family rarely are more than 10 m apart⁵, whereas sirening and alarm trills are audible for about 800 m, more than twice the diameter

Table 1 Estimated audible ranges of adult Kloss's gibbon vocalisations to a man on the ground

Vocalisation	Context	Estimated audible range (m)
1 Hoo	Detection of langurs, other gibbons, or humans	50-100
2 Howl	Detection of langurs, other gibbons, or humans	100-150
3 Whup	During female song bouts. Occasionally precedes male song, sometimes emitted on detecting humans	400
4 Whistle	Precedes male song; occurs during female song bouts and is occasionally emitted upon detecting humans	50-100
5 Whoop	Precedes female song	400
6 Sirening	Detection of a human	800
7 Alarm trills	Detection of a human	800
8a Male song	Begins spontaneously or on hearing other males singing	500-700
8b Female song	Begins spontaneously, on hearing other females singing or on encountering another female at territorial boundary	800
9 Quivering squeal	Males fighting (emitted by males)	50-100
10 Whistle-howl	Males fighting (emitted by females)	50-100

Note that six of the ten call types may occur when a predator (human) is detected but only sirening and alarm trills are restricted to this context.

of an average territory (x diameter = 350 m). Since softer alarm calls in the Kloss's gibbon vocal repertoire (Table 1) are sufficient to signal the immediate family, we suggest their loud alarm calls evolved by selection favouring gibbons who warn neighbours of danger.

Evidence on gibbon genealogy indicates a high probability that gibbons occupying neighbouring territories are close relatives. Four instances of settling by Kloss's gibbons in Siberut were observed. In 1972 a male was apparently being aided by his parents to establish a territory contiguous with theirs, and a female consorted⁶ and finally mated (R.L.T., in preparation) with a male holding a territory contiguous to that of her parents. Two additional cases of settling were documented in 1973–74. In one a male was aided by his parents to establish a territory situated 150 m from theirs, and in another a female was accompanied by her parents while being courted by a male whom she eventually settled with in a territory contiguous with that of her parents (R.L.T., in preparation). In each of the three cases where a successful breeding pair was formed, at least one of the pair was the offspring of the occupants of an adjacent or nearby territory. While spatial limitations and the presence of floating males in the population⁶ indicate that not all gibbons settle within audible range of their parental families, these observations show that at least some do. In the only other case of settling by a wild gibbon which has been observed, Aldrich-Blake and Chivers¹² report that a male siamang (*H. syndactylus*) established a territory contiguous with that of his parents on reaching physical maturity. Although siamangs do not have specialised long-distance alarm calls, they do emit a distinguishable rendition of their loud territorial call when alarmed by a potential predator¹³.

In each case cited above a mature offspring settled within audible range (for long-distance alarm calls) of parents and younger siblings, with each of whom it shares a coefficient of relationship of 0.5. Theoretically, in such a socio-genealogical framework, long-distance alarm calls that do not appreciably lower the individual fitness of a signaller could increase the inclusive fitness of neighbouring relatives and thus be favoured by kin selection^{4,14,15}. The fact that every species of gibbon which has been studied produces some kind of distinguishable long-distance alarm call^{16–18} suggests this could be true for all species of hylobatids. The apparently high probability of close genetic relatedness among neighbouring Kloss's gibbons makes it unnecessary, in our opinion, to invoke hypotheses of reciprocal altruism¹⁹ or selfish manipulation^{20,21} in attempts to explain the evolution of alarm calls in this species.

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Role of sister chromatid exchanges in chromatid aberration formation

MECHANISMS of formation of chromatid aberrations are not fully understood. The involvement of chromosome breakage and reunion¹ or exchange² has been suggested. DNA repair is a possible cause of aberrations and exchanges^{3–5}, but a causal relationship between the two phenomena is not clear^{6,7}. It is not known whether chromatid deletions derive from unrepaired chromatid breaks or from incomplete exchanges between sister chromatids. The origin of chromatid deletions can be studied by examining whether or not they occur just at the points of sister chromatid exchanges (SCE) (ref. 6). Differential labelling of sister chromatids with 5-bromodeoxyuridine (BUdR) has shown that X-ray-induced chromatid deletions are only infrequently associated with SCE, indicating that most chromatid deletions are not incomplete SCE but simple chromatid breakage⁸. On the other hand, the parallel distribution of SCE and chromosome aberrations induced by two different hydrocarbon carcinogens, 7,12-dimethylbenz (a) anthracene and 7,8,12-trimethylbenz (a) anthracene has been reported, suggesting that aberrations are incomplete SCE (ref. 9). The results presented here indicate that almost no chromatid deletions or gaps induced by irradiation of BUdR-substituted chromosomes with visible light represent incomplete SCE.

When Chinese hamster ovary (CHO) cells cultured for two rounds of replication in the presence of BUdR were

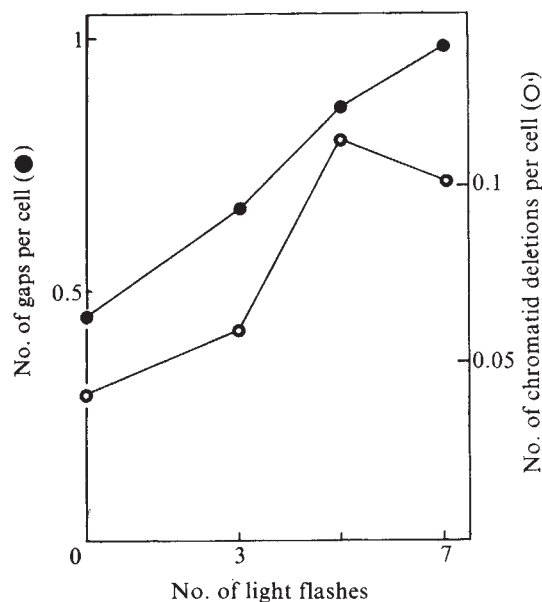


Fig. 1 Frequencies of chromatid deletions and gaps plotted against the number of light flashes. CHO cells were cultured in the dark in McCoy's 5A medium containing 15% foetal calf serum and 10 μ M BUdR for 24 h. The cells were then exposed to visible light from an electric photographic flash unit placed 10 cm from the cell layer: the light was filtered through a 5-mm acryl-resin plate. Colcemid (final concentration 10^{-7} M) was added to the culture 2 h before collecting the metaphase cells. The sample was therefore irradiated with visible light in the G₂ phase. Chromosome specimens were prepared and the slides were stained by the Giemsa^{12,13} or modified 33258 Hoechst-Giemsa methods¹⁴. 100 cells were observed for each light intensity.

exposed to visible light from a photographic electric flash unit, chromatid aberrations, excluding exchange types, and SCE, were induced frequently. Using such cells, we investigated whether or not chromatid deletions occurred just at the points of SCE.

The frequencies of chromatid deletions and gaps increased with increasing light flashes, or intensity of visible light (Fig. 1). The number of SCE in each chromosome was also in proportion to the light intensity and the relative size of chromosomes (Fig. 2) (ref. 12). Chromatid aberrations and SCE observed may have resulted from DNA strand breaks induced by photolysis of BUdR-containing chromosomes, for photolysis of BUdR-incorporated DNA leads to polynucleotide strand breaks¹⁰.

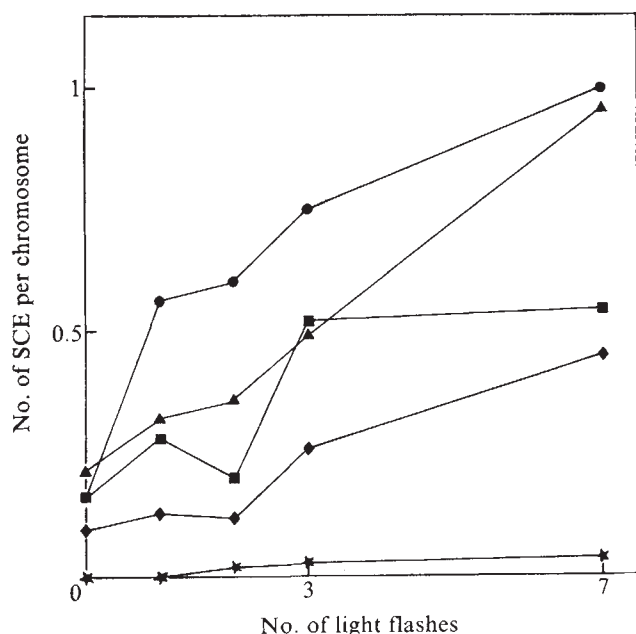


Fig. 2 Frequency of SCE plotted against the number of light flashes. SCE were scored for the largest (●), the second largest (▲), the third largest (■), medium-sized (◆) and small (★) chromosomes, respectively. Each data point represents the analysis in more than 25 chromosomes.

Chromatid aberrations were scored according to whether aberrations were accompanied by SCE. Chromatid deletions were classified into three types according to the position of distal segments (Fig. 3). Only one out of 42 chromatid deletions and 22 out of 632 gaps observed contained SCE at the breakpoints (Table 1). These results indicate that very few chromatid deletions or gaps are incomplete exchanges.

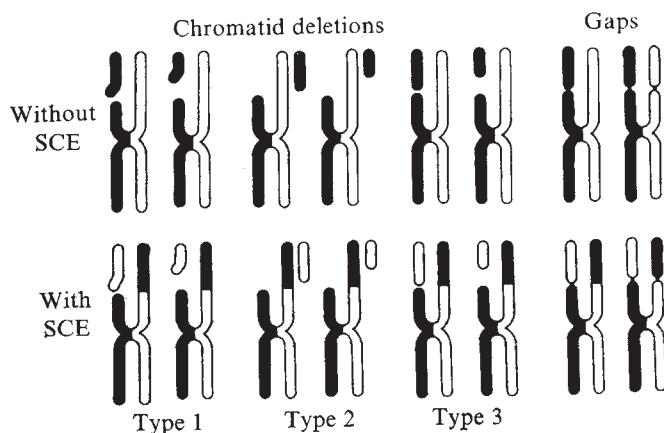


Fig. 3 Schematic representation of three types of chromatid deletions and gaps scored. Type 1, slightly displaced distal segment; type 2, completely displaced distal segment; type 3, non-displaced distal segment.

Table 1 Chromatid deletions and gaps with or without SCE at breakpoints

Chromatid type*	Chromatid deletions†						Gaps	
	Type 1	Type 2	Type 3	Total			+	-
BB	1	4	24	29			440	
BT	0	0	1	1			22	
Total	1	2	9	12			170	
	0	0	1	1	41	22	610	

+, With SCE; -, without SCE.

* BB, BUdR bifilarly substituted; BT, BUdR unifilarly substituted.

† Classified according to Fig. 3.

This is consistent with the observations for X-ray-induced chromatid deletions⁸. The lesions and/or subsequent processes leading to chromosome aberrations might be different from those involved in sister chromatid exchanges.

Table 1 also implies that bifilarly BUdR-substituted chromatids are more than twice as sensitive to light flashes as are unifilarly substituted chromatids. Equal sensitivity has been found to X rays in unifilarly- and bifilarly-substituted chromosomes, both of which are more sensitive than unsubstituted chromosomes^{8,11}. This apparent difference in sensitivity might reflect some fundamental distinctions between the two types of radiation for production and repair processes of DNA strand breaks responsible for aberrations in BUdR-containing chromosomes, although it is difficult to speculate on the nature of the distinction.

The results presented here indicate that, whatever the mechanisms of formation of chromatid aberrations or SCE, the relationship of SCE to chromatid breaks depends on the type of agent used to damage the chromosomes.

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Interferon suppresses the transition of quiescent 3T3 cells to a growing state

PAUCKER, Cantell and Henle reported that crude mouse interferon preparations depressed multiplication of suspended L cells¹. Since then evidence has accumulated indicating that interferon molecules which inhibit multiplication of virus also inhibit the growth of cells in culture²⁻⁹. Interferon was shown to suppress incorporation of ³H-thymidine into cellular DNA in various systems—for example, release from a double thymidine block in synchronised L 929 cells¹⁰; activation of mouse spleen lymphocytes by phytohaemagglutinin (PHA) or allogeneic lymphocytes¹¹; and in steady-state growth of cultured mouse leukaemia L 1210 cells¹² in a chemostat. However, there has been no direct evidence to distinguish between the effect of interferon on the rate of initiation of DNA synthesis (entry into the S phase) and that on the rate of DNA synthesis in the S

phase, and it is uncertain which step of the cell cycle is interfered by interferon. It is well known that most cells in cultures of confluent or serum starved mouse 3T3 stay in the early G_1 or G_0 stage of the cell cycle, and an addition of serum to these quiescent cells stimulates their growth, leading to DNA synthesis and cell division^{13,14}. We describe here a suppressive effect of interferon on the transition from the quiescent to the growing state in BALB/c 3T3 cells. The data indicate that interferon suppresses the initiation of DNA synthesis.

Interferon preparations used were obtained from the supernatant of monolayer culture of mouse L cells inoculated with Newcastle disease virus and purified by column chromatography using DEAE-Sephadex and CM-Sephadex (2×10^7 units per mg protein) (ref. 15). Interferon titres were expressed in international mouse reference units.

Confluent and serum-starved quiescent BALB/c 3T3 cells were transferred to fresh medium containing 10% foetal bovine serum and ^3H -thymidine with or without interferon, and the proportion of cells with labelled nuclei (labelling index) was determined as a function of incubation time by autoradiography (Fig. 1a). The labelling index shows the proportion of cells which have entered the S phase. The cells synthesising DNA began to appear after a 12-h lag period either in the presence

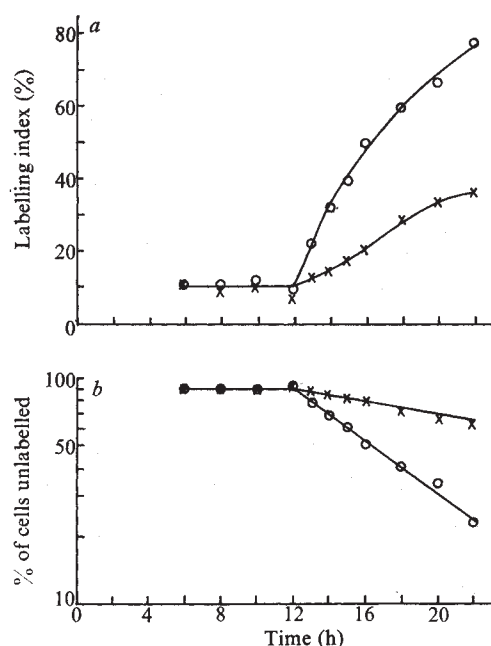


Fig. 1 Effect of interferon on the entry into the S phase after stimulation of quiescent BALB/c 3T3 cells with addition of fresh medium containing 10% serum. BALB/c 3T3 cells¹⁶ (from Flow Laboratories) were plated at 4×10^4 cells on to 15-mm plastic coverslips (Wako Pure Chemical Industries) submerged in each well of Linbro multi-dish tray (Linbro 24-16-TC) containing 1 ml of Dulbecco's Modified Eagle's Medium (GIBCO) with 10% foetal bovine serum (Microbiological Associates), and grown at 37 °C in a 10% CO_2 atmosphere (v/v) until confluent. Four days after they were seeded, cell monolayers ($\sim 2 \times 10^6$ cells per plate) were washed with phosphate-buffered saline, and added 0.5 ml of serum-free Modified Eagle's Medium. After incubation for further 12 h, the medium was discarded and replaced by 0.5 ml of fresh medium containing 10% foetal bovine serum and ^3H -thymidine ($1 \mu\text{Ci ml}^{-1}$; $2 \mu\text{M}$ thymidine) with or without addition of interferon (1×10^4 units per ml). At the times indicated, coverslips were removed and cells were washed with phosphate-buffered saline and extracted with 5% perchloric acid, rinsed with ethanol and air dried. Coverslips were mounted on to glass microscope slides and they were dipped into SAKURA autoradiographic emulsion NR-M2 (diluted with an equal volume of distilled water). After exposure for 7 d at 4 °C, autoradiographs were developed, stained with Giemsa solution, and the proportion of cells with labelled nuclei was determined. *a*, Proportion of cells entering into the S phase (labelling index). *b*, Proportion of cells remaining in the G_1 phase (100 minus labelling index). $\circ$, No interferon; $\times$, Interferon added.

or absence of interferon, but the rate of increase of the DNA-synthesising cells was reduced when interferon was present in the culture; that is, interferon reduced the frequency of the initiation of DNA synthesis, but it did not shift the point where cells began to enter into the S phase. The result is reminiscent of the fact that, although serum concentration determines the rate at which cells enter the S phase, the length of the lag period is independent of the serum concentration¹⁷.

Smith and Martin have proposed a new theory on the cell cycle, in which a cell's life is divided into two fundamentally different parts: A-state and B-phase¹⁸. The B-phase is a determinate one including the S, G_2 and M phases in addition to a part of the G_1 phase. On the other hand, the A-state is rather indeterminate. After mitosis the cell enters the A-state and remains for any length of time with a constant probability of leaving this state ('transition probability'). Brooks has analysed the regulation of the fibroblast cell cycle by serum according to the above theory, and showed that serum factors control the rate of transition from the A-state to the B-phase^{17,19}.

Figure 1b replotted from Fig. 1a shows the proportion of cells remaining in the G_1 phase (cells unlabelled) indicating that after 12-h lag period the rate of decrease of the G_1 cells either in the presence or absence of interferon followed first-order kinetics. In this experiment the transition rate constant was 0.13 h^{-1} in interferon-free control cultures, while in those with added interferon (1×10^4 units per ml) it was 0.036 h^{-1} . The rate constant estimated here is not exactly the same as 'transition probability' (P) defined by Smith and Martin¹⁸, since in our experiments a small portion of the quiescent 3T3 cells was not in the G_0 or G_1 phase (A-state). It is closely related to P , however, because the rate of decrease of unlabelled cells after a lag period followed first-order kinetics. Thus, it can be said that interferon reduced the 'transition probability' from the A-state to the B-phase. Furthermore, the transition rate constant declined proportionally to the amount of interferon added (Fig. 2).

The effect of interferon on the transition from the G_1 phase to the S phase was examined when interferon was added at various times following serum stimulation (Fig. 3). When added at zero time or 4 h, the suppressive effect of interferon on the transition was almost the same, but after 4 h it declined, and at the time when the cells began to enter into the S phase the addition of interferon did not show any effect on the initiation of DNA synthesis. A certain period may be required for the expression of the suppressive activity of interferon, or interferon may not have any effect on the cells already committed to DNA synthesis.

Although our interferon preparations are not completely pure, the following results strongly indicate that interferon itself is responsible for the observed effect. First, when the interferon preparations were heated at 100 °C, their antiviral

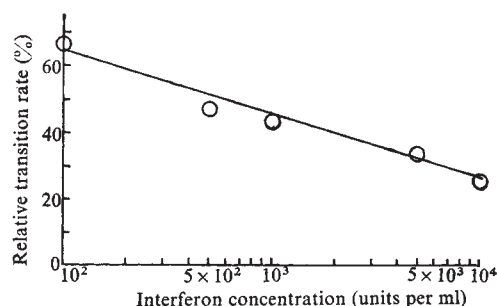


Fig. 2 Relative transition rate as a function of interferon dose. Quiescent and serum starved BALB/c 3T3 cells were stimulated by the addition of fresh medium containing 10% foetal bovine serum as described in Fig. 1. Various doses of interferon were added at zero time. Coverslips were removed at 8, 10, 12, 14, 16, 18, 21 and 24 h, respectively, and processed by autoradiography. Autoradiographs were exposed for 12 d. The proportion of cells remaining in the G_1 phase was plotted semilogarithmically as a function of time and the transition rate constant was obtained. In interferon-free control cultures it was 0.10 h^{-1} . Ordinate indicates the relative transition rate to the control.

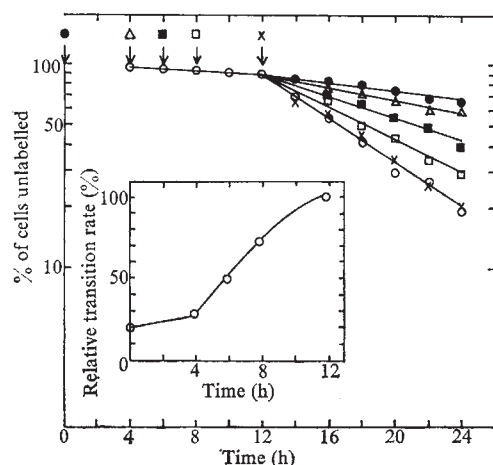


Fig. 3 Effect of addition of interferon at various times during stimulation on the rate of entry into the S phase. Quiescent and serum-starved BALB/c 3T3 cells were stimulated by the addition of fresh medium containing 10% foetal bovine serum, and interferon (1×10^4 units per ml) was added at the times indicated. Coverslips were removed and processed by autoradiography. Autoradiographs were exposed for 7 d. The inset to the figure describes the relative transition rate as a function of the time when interferon was added. In interferon-free control cultures the transition rate constant was 0.13 h^{-1} .

activity was reduced to less than 1%, but on heating in the presence of sodium dodecyl sulphate (SDS), about one-third of it survived (Table 1). The suppressive effect of interferon on the transition from the quiescent to the growing state of the cells was also lost by heating, but preserved if heated in the presence of SDS, in parallel to the antiviral activity see the dose-response curve in Fig. 2). Similar results have already been reported by Stewart *et al.*⁹. Second, both activities were lost on trypsin digestion of the interferon preparations (Table 1). Third, the L cell interferon preparations, used at 1×10^4 units per ml, did not exert any effect on the serum activation of the serum-starved quiescent BHK cells (data not shown), conforming to the well-known species specificity of interferon action.

Interferon molecules may suppress certain cellular events in the G_1 stage of the cells, since the values for the transition rate

Table 1 Antiviral and anticellular activities of interferon after heating with or without SDS, and trypsin digestion

Treatment of interferon*	Antiviral activity† survival (%)	Anticellular activity† Transition rate constant (h^{-1})	Relative transition rate (%)
No treatment	100	0.015	20
-SDS, 100°C , 1 min	0.5	0.038	51
+SDS, 100°C , 1 min	34	0.013	18
-SDS, 100°C , 4 min	0.1	0.043	58
+SDS, 100°C , 4 min	34	0.013	18
+Trypsin, 37°C , 30 min	0.04	0.051	69
Without interferon	—	0.074	100

*Small tubes containing 20 μl of L cell interferon (1×10^7 units ml^{-1} , 0.5 mg protein ml^{-1}) in phosphate-buffered saline were prepared. They were constituted to contain 1% SDS-0.1 mM dithiothreitol or 0.1% trypsin (Sigma) when indicated. After treatment, 80 μl of Dulbecco's Modified Eagle's Medium containing 10% foetal bovine serum was added.

†Antiviral activity was assayed by the method described by Suzuki *et al.*²⁴. Reduction of incorporation of ^3H -uridine into RNA of vesicular stomatitis virus in actinomycin-D-treated mouse L cells was estimated.

‡Effect of interferon on the transition from a quiescent to a growing state in BALB/c 3T3 cells was observed as described in Fig. 1. Non-treated interferon was used at 5×10^4 units per ml.

constant ('transition probability') reflect the cellular activities during the transition from a quiescent to a growing state (G_1 phase or A-state)^{19,20}. The transition is known to be accompanied with a complex set of cellular changes (see ref. 21), termed the "pleiotypic control" by Hershko *et al.*²². It was reported that interferon elevates the cellular levels of cyclic AMP in the cultures of L 929 cells²³. The suppressive effect of interferon on the initiation of DNA synthesis may be resulted partly from the failure in falling of cyclic AMP levels. We have recently observed the effect of interferon on the growth rate, labelling index, fraction of labelled mitosis and mitotic index of BALB/c 3T3 cells in exponentially growing culture. The data showed that the duration of the G_1 phase was only affected by the addition of interferon (in preparation).

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Inhibition of lymphocyte-induced angiogenesis by isolated chondrocytes

LYMPHOCYTE-induced angiogenesis (LIA) has been introduced by Sidky and Auerbach¹ as a quantitative and sensitive assay for measuring cell-mediated immunity *in vivo*. In this assay immunocompetent lymphocytes are injected intradermally into allogeneic or semiallogeneic mice. A local graft versus host (GVH) reaction is therefore induced, resulting in new blood vessel formation. As early as the second day post-injection, this effect could be observed in a dose-response fashion dependent on the number of cells injected. The relationship of lymphocyte-induced angiogenesis to tumour-induced angiogenesis mediated by tumour-angiogenesis factor (TAF)² remains theoretical. Folkman and others have demonstrated inhibition of TAF-induced angiogenesis by cartilage fragments³ or extracts⁴. In this study we show that isolated chondrocytes can inhibit the angiogenesis induced by lymphocytes *in vivo*. Their ability to do so depends on the ratio of chondrocytes to lymphocytes used.

LIA assays were performed in 8-10-week-old female (CFW $\times$ C57Bl/Sn)F₁ hybrid mice. Recipients were injected intradermally with 0.1 ml of Hanks solution containing from 1.0×10^6 - 4.0×10^6 viable lymphoid cells obtained from 8-10-week-old parental CFW female donors by mechanical disruption of the spleen in a loose-fitting glass homogeniser. Control

injections contained 4×10^6 viable syngeneic F_1 spleen cells or 4×10^6 parental CFW thymocytes.

Chondrocytes were obtained from cartilaginous epiphyses of long bones dissected from 6–10-d-old CFW mice. Cartilage fragments were digested with a mixture of 0.25% collagenase, 0.25% trypsin and 0.05% streptodornase in Hanks solution⁵. Cells liberated from the matrix were rinsed three times in Hanks solution with 30% calf serum and then twice in Hanks solution alone. Viability, estimated with the Trypan blue exclusion test, was greater than 85%.

To measure the effect of chondrocytes on lymphocyte-induced angiogenesis, variable numbers of viable chondrocytes (from 0.2×10^6 to 2.0×10^6) were mixed with 4×10^6 viable syngeneic or parental spleen cells in a total volume of 0.1 ml of Hanks solution to obtain a chondrocyte–lymphocyte ratio of 1 : 20, 1 : 10, 1 : 5 or 1 : 2 respectively. Control injections consisted of chondrocytes only or of spleen cells mixed either with freeze-thaw killed chondrocytes or with viable kidney cells obtained from adult CFW donors by digestion with the enzymatic solution used for chondrocyte isolation. Trypan blue (2–3 drops) was added per 1 ml of each inoculum injected to facilitate the subsequent location of the injection site. To eliminate the possibility of individual recipient variations in reactivity, each animal received up to 8 different inocula, tested in a coded manner. The animals were killed after 3 d and the skin carefully dissected from the underlying muscles to expose its inner surface under a dissecting microscope using a green filter. All blood vessels directed towards the injection site and contrasting with the background vasculature because of their contorted shape and pathways were scored according to the criteria proposed by Sidky and Auerbach¹. The number of vessels evoked by intradermal injection of parental spleen cells compared with other cell types is shown in Table 1.

The lymphocyte-induced angiogenesis assay used in the present study is a lymphocyte-dependent at least initially, when there is lymphocyte contact with the host antigens. Specific recognition by competent cells must first take place to induce

neovascularisation, since the angiogenesis evoked by syngeneic F_1 spleen cells is negligible. Also, angiogenesis induced by parental thymocytes is weaker than that induced by parental spleen cells. Lymphocytes stimulated by host antigens may release angiogenetic factors or, alternatively, these factors could be products of tissue destruction resulting from an immunological reaction. Lymphocytes also produce substances chemotactic for granulocytes^{6,7} and polymorphonuclear leukocytes have been shown to evoke angiogenesis in the rabbit cornea⁸. However elicited, the stimulatory effect on capillary growth is related to the number of the competent lymphocytes injected.

These results show that chondrocytes, in appropriate concentration, inhibit the angiogenesis induced by lymphoid cells. At a chondrocyte–lymphocyte ratio of 1 : 10 this effect is most marked, reducing the intensity of the angiogenetic response evoked by 4×10^6 spleen cells to a level similar to that produced by 2×10^6 spleen cells. This effect is specific for viable chondrocytes since neither killed chondrocytes nor kidney cells show this inhibitory effect on angiogenesis.

Isolated chondrocytes produce matrix when injected into recipient animals⁹ and some of their products could affect endothelial cell growth. Small cationic proteins with protease-inhibiting properties have been shown to be responsible for the resistance of cartilage to invasion by vascular mesenchymal cells^{9,10}. Cartilage extract inhibits the growth of endothelial cells *in vitro*¹¹. Cartilage fragments or extracts inhibit TAF-induced neovascularisation *in vivo*^{3,4} and cartilage tumours are the least vascularised of all tumours tested¹². The inhibitory effect of chondrocytes thus seems to be similar regardless of the type of stimulus used to induce neovascularisation. The stimulatory factors may be produced by lymphocytes as lymphokines or their production may be lymphocyte dependent. In practice, tumour-induced and lymphocyte-induced angiogenesis are indistinguishable¹³. TAF has been isolated from tumour cells² but in many tumours the extent of lymphocytic infiltration is rather high¹⁴. Tumours do not grow well in the

Table 1 The number of blood vessels evoked in (CFW $\times$ C57B1/Sn) F_1 mice by intradermal injection of parental or syngeneic lymphoid cells mixed with non-lymphoid parental or syngeneic cells

Cells injected	No. of vessels (mean $\pm$ s.d.)	No. of injections	Conclusion
Parental spleen cells			
4×10^6	27.75 ± 5.34	52	Angiogenesis all results differ
2×10^6	20.66 ± 4.19	12	
1×10^6	15.83 ± 3.24	12	
4×10^6 parental spleen cells mixed with parental chondrocytes in ratio			
20:1	24.76 ± 7.03	20	Weak inhibition of angiogenesis
10:1	18.96 ± 6.08	30	Marked inhibition of angiogenesis
5:1	29.16 ± 4.70	6	No inhibition
2:1	35.75 ± 6.19	4	Stimulation
4×10^6 parental spleen cells mixed with parental kidney cells obtained enzymatically in ratio			
10:1	26.58 ± 7.12	12	No inhibition
5:1	28.66 ± 5.24	6	
4×10^6 parental spleen cells mixed with killed parental chondrocytes in ratio			
10:1	25.86 ± 7.62	6	No significant inhibition
Parental thymocytes 4×10^6	12.15 ± 7.62	13	Not different from 1×10^6 spleen cells
4×10^6 syngeneic (F_1) spleen cells	6.21 ± 2.65	47	Control
4×10^6 syngeneic spleen cells mixed with parental chondrocytes in ratio			
10:1	5.35 ± 3.81	6	Not different from control
or with syngeneic chondrocytes in ratio			
5:1	14.16 ± 2.63	6	Weak angiogenesis
Syngeneic chondrocytes 8×10^6	4.00 ± 1.41	4	Control

Significance at $P < 0.05$ level estimated by Student's t test.

anterior chamber of the eye or in perfused organs¹⁵ where the access of lymphocytes to tumour cells is prevented. A rare spontaneous tumour growth in nude mice¹⁶ could support this assumption, favouring Prehn's immunostimulation theory¹⁷.

We found that at higher chondrocyte-lymphocyte ratios (1 : 5 or 1 : 2) an increase of angiogenesis rather than inhibition was seen, suggesting that an immunological reaction may be occurring against tissue-specific antigens on the chondrocyte surface. It has been shown that syngeneic rat chondrocytes can evoke an immune response suggesting that these chondrocytes possess a sequestered antigen¹⁸⁻²⁰. The same is probably also true for murine chondrocytes.

Therefore, when chondrocytes and lymphocytes are mixed, two different processes may occur. At low ratios primarily an angiogenesis-inhibiting process occurs, but at higher ratios this is overcome by the increased production of lymphocyte-angiogenic substances directed against chondrocyte-specific antigens which increases new blood vessel formation, rather than decreases it as would be expected.

The finding that viable chondrocytes can inhibit new blood vessel formation has some morphogenetic implications for osteogenesis. Capillaries from perichondrium cannot penetrate cartilage anlagen of long bones. When a number of chondrocytes die in the ossification centre, the local concentration of the inhibitory substance decreases, which might permit localised invasion of capillaries of the periosteal bud and thus initiate osteogenesis.

In addition, we have tested the effect of 1 M HCl-guanidine extract of human costal rib cartilage (obtained from biopsy) on mouse endothelial cell growth induced by local injection of allogeneic murine lymphocytes. This extract also inhibited endothelial cell growth (unpublished) showing that the cartilage inhibitory effect can act across the xenogeneic barrier.

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Human smooth muscle autoantibodies reacting with intermediate (100 Å) filaments

THE specificity of human smooth muscle autoantibodies (SMA) is known only in chronic active hepatitis where SMA are of anti-actin type^{1,2}. In other diseases there seem to be SMA of additional specificities³⁻⁵. Anti-actin antibodies react with microfilaments of cultured cells^{6,7}. We

describe here two human SMA-positive sera which react in indirect immunofluorescence microscopy (IFL) with other cytoskeletal structures, intermediate (100 Å) filaments. Such filaments are especially abundant in smooth muscle cells^{8,9} but occur also in other cells including fibroblasts¹⁰.

The SMA-positive sera were obtained from two cancer patients and reacted typically with smooth muscle tissue by indirect IFL technique¹¹ but could not be neutralised with purified actin. These sera reacted also with intercalated disks and the periphery of rat cardiac myofibrils. A similar

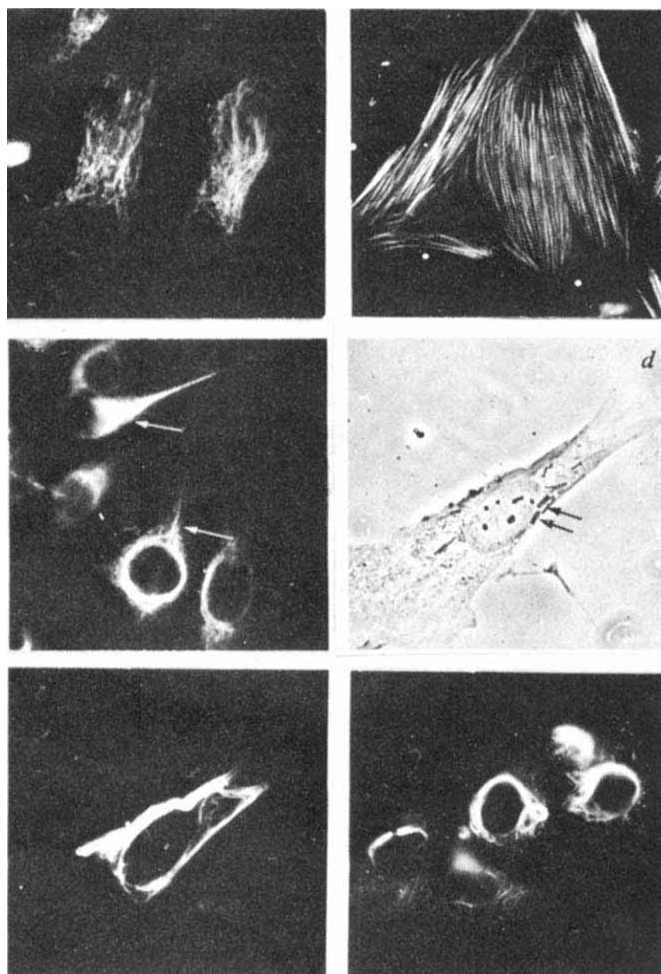


Fig. 1 Indirect immunofluorescence (IFL) staining of cultured cells with two human sera, one containing smooth muscle antibodies (SMA) to intermediate filaments (*a* and *c-f*) and the other to actin (*b*). Both sera had a SMA titre of 5,000 determined by indirect IFL using unfixed cryostat sections of rat stomach and kidney as substrates. To visualise human antibodies FITC-conjugated sheep anti-human immunoglobulin (NBL, Solna, Sweden, F/P molar ratio 2.9) was used. Human embryonic skin fibroblasts cultivated in RPMI 1640 + 10% foetal calf serum were used in their 10th to 25th passage *in vitro*. Mouse neuroblastoma cells were cultivated in MEM + 10% foetal calf serum. Cells were grown on cover slips for 48 h before fixation for 10 min in -20 °C acetone. *a*, Reaction of SMA of anti-intermediate filament type with fibroblasts. Note the fine fibrillar network in the cytoplasm. *b*, Reaction of SMA of anti-actin type with fibroblasts. The typical filamentous staining is seen. *c*, Reaction of SMA of anti-intermediate filament type with mouse neuroblastoma cells. Note the perinuclear bundles (arrows). *d*, A fibroblast, treated with vinblastine sulphate for 4 h (Lilly, 10 µg ml⁻¹), visualised in phase contrast microscopy. Tubulin containing paracrystals, induced by the treatment are indicated by arrows. *e*, The same cell as in (*d*) stained by SMA of anti-intermediate filament type. Coiling perinuclear bundles of fluorescence are seen. The paracrystals are not visualised. *f*, Reaction of SMA of anti-intermediate filament type with fibroblasts treated with demecolcine for 12 h (Colcemid, Ciba, 0.1 µg ml⁻¹). Thick coiling fluorescent bundles are seen at the perinuclear area also after this treatment.

staining pattern was recently reported by Lazarides and Hubbard¹² who used an experimental antiserum to 'desmin', a subunit protein of intermediate (100 Å) filaments.

In human embryonic skin fibroblasts our SMA-sera stained a fine fibrillar cytoplasmic network by IFL (Fig. 1a). This fluorescence pattern clearly differed from the microfilament staining produced by SMA of anti-actin type (Fig. 1b) and could not be inhibited with purified actin. A preparation of intermediate (100 Å) filaments isolated using the method of Cooke⁸ completely inhibited both the fibrillar staining of fibroblasts and the staining of smooth muscle and cardiac tissues. To identify the fibrillar structures found in cultured fibroblasts by IFI we treated cells with cytochalasin B, vinblastine and demecolcine (Colcemid). These drugs induce typical alterations in the cellular cytoskeleton^{10,13}. Cytochalasin B characteristically abolished the filamentous staining obtained with anti-actin antibodies but the fibrillar staining was not affected. Treatment with vinblastine resulted in the formation of paracrystals which are known to contain tubulin¹⁴. These remained unstained by IFL (Fig. 1d, e). Instead, in cells treated either with vinblastine or demecolcine perinuclear coiling bundles were seen (Fig. 1e, f). Such bundles, induced by microtubule-disrupting drugs, consist of intermediate filaments¹⁰. This was confirmed in our study by electron microscopy of demecolcine- (Fig. 2) and vinblastine-treated cells.

When cultured mouse neuroblastoma cells¹⁵ were stained by IFL, bright perinuclear bundles were seen (Fig. 1c). The bundles were similar to those observed by Jorgensen *et al.*¹⁶ who used antibodies specific for a subunit protein of neurofilaments. The reaction of our sera with neuroblastoma cells corroborates the recent suggestions that neurofilaments and intermediate filaments are identical^{16,17}.

Our results show that treatment of cells with drugs characteristically altering intracellular organisation offers a method in studies of autoantibodies reacting with cytoskeletal structures. Using this approach we have found that some SMA react with autoantigens located in intermediate filaments. Thus far only one component of the intermediate filaments has been described, desmin¹² or skeletin⁹, having a molecular weight of between 54,000 and

55,000. Whether our sera contain antibodies against this protein is not yet known.

Intermediate filaments have been studied mainly by electron microscopy^{10,18,19}. Human autoantibodies offer an additional tool to study the function and suggested identity of these filaments in different cells.

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Myogenic defect in human muscular dystrophy

THE aetiology of muscular dystrophy has been considered to be either myogenic or neurogenic¹. Much of the evidence has been obtained from studies of muscle in tissue culture using animal² or human³ sources. Although results have often been equivocal^{4,5} the most recent studies have failed to show any morphological differences between normal and abnormal muscle^{3,6,7}. Hence, it could be argued that the primary defect is not in the muscle but rather may be in the nerve which innervates the muscle⁸. But, almost all of these studies have involved techniques based on explants of small muscle pieces, which muscle fragments then typically require one week for outgrowth from the tissue pieces and a further week or two before differentiated function (myotube formation) is seen^{3,6}. For expression of maximally differentiated cultures (cross striations and spontaneous contractions) it has been presumed that innervation may be required, since contractions are rarely found and are essentially seen only in the presence of added nerve cells⁹. We have recently described a new technique for establishing fully differentiated adult muscle cultures from mononucleated cells and have found spontaneous contractions without the requirement for nerve cells⁸. Using this method, we have compared the maturation of muscle from the Duchenne (X-linked recessive) form of muscular dystrophy with appropriate control cases, and have found an abnormality in cellular behaviour restricted to the Duchenne material.

Table 1 shows the results from cultures of nine dystrophic patients and nine controls and demonstrates that in

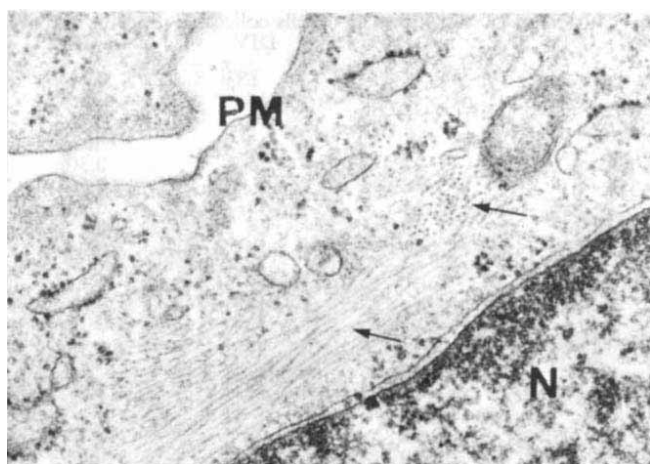


Fig. 2 An electron micrograph of a cell treated with demecolcine ($0.1 \mu\text{g ml}^{-1}$) for 12 h. For electron microscopy the cultures were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), postfixed in 1% osmium tetroxide and embedded in Epon 812. Thin sections were transversally sectioned from cultures and post-stained with uranyl acetate and lead citrate. Electron microscopy was done at the Department of Electron Microscopy, Helsinki University. Bundles of filaments, 10 nm in diameter, are seen perinuclearly both tangentially and transversally sectioned (arrows). N, Nucleus; PM, plasma membrane. $\times 39,000$.

each case the abnormal formation of clustered cells occurs before the appearance of myotubes. It also demonstrates that the level of creatine phosphokinase (CPK) specific activity is generally lower than in controls. The first sign of abnormality can be detected as early as four days after plating the cells and consists of areas where the cells are clustered together in close proximity (Fig. 1a) rather than being more uniformly distributed in a monolayer (Fig. 1b). Some of the cells in the clusters had a superficial resemblance to fibroblasts, but more careful examination revealed that they were quite atypical in their morphology. The nuclei of these cells as well as the perinuclear cytoplasm seemed to be increased in size and hence might be termed hypertrophic, as previously described^{9,10}. They also have longer processes (Fig. 1a). There is, however, a proportion of myoblasts which fuse in apparently normal fashion.

With further differentiation there are a certain number of refractile myotubes but their distribution is again abnormal in that they typically span the gap between two of the multilayered cell clusters which are by now several cells thick (Fig. 1c) and thus give the appearance of a pyramidal form of multicellular complex when one focuses up and down along their margins. Of course, the photographs do not directly reveal this third dimensional consideration. The clear areas on the surface of the culture plate between the hypertrophic clusters are still prominent at this stage, which is again quite different from the normal muscle (Fig. 1d) or from the other diseases referred to previously. Within these cases there was no obvious correlation between the degree of connective tissue found in the biopsy and the degree of cluster formation; quite the contrary—the patient (M.C.) with a massive excess of fibroblast infiltration took the longest time (Table 1) to exhibit the abnormality.

Although there have been various comments on the behaviour of dystrophic muscle in culture, other authors have not described these particularly early abnormal events during the maturation sequence. It is thus quite different from the normal sequence whereby single myogenic cells

fuse into multinuclear myotubes and then differentiate into fully refractile muscle straps¹¹. Those techniques which initiate cultures from small explants of tissue pieces have a requirement for cell migration from the initial explants, before the myoblasts can begin to fuse and form myotubes. This difference in the methods for starting the cultures may explain in part why explant techniques obscured these early events. But, the technique which starts with single cells does not select for cells which must migrate out of the explant before they can differentiate into myotubes. A closer approximation to starting cultures with single cells has been the technique which begins with smaller 'muscle cell pieces' ($60 \times 25 \mu\text{m}$) each of which contains a few nuclei that grew initially by 'budding'¹². This technique revealed some abnormal intracytoplasmic inclusions but the abnormalities in cellular distribution and myotube formations were not described¹³.

Concerning the primary metabolic lesion, there are several possible defects in cellular metabolism which give rise to the abnormal muscle development (for example, membrane alterations^{7,14,15}). But it should also be considered that the genetic defect may involve an abnormality of control gene which normally initiates a specific sequence (for example, during the orientation of thick and thin filaments) during cellular differentiation¹⁶.

It should not yet be concluded that the abnormalities are diagnostic for Duchenne dystrophy. The origin of the cells may be an abnormal form of fibroblastic connective tissue. They may also be aberrant precursors of myoblasts, however.

The advantages of the current technique are that one has live cells which can be collected for specific metabolic assays, that certain cells display an unusual form of cell-cell interactions, that the cells within the unusual clusters do not seem to resemble normal fibroblastic or epithelial morphologies¹⁷ and that the complete sequence of muscle maturation¹¹ can be observed and sampled at appropriate developmental times. Further, since these abnormalities

Table 1 Comparative data on dystrophic muscle biopsies and controls run in the same culture conditions

Patient	Age (yr)	Diagnosis	No. of Petri plates	Clusters at DIV	Myotubes at DIV	Cells collected DIV	Specific activity of CPK
J.W.*	5	Duchenne	5	7	8	15	0.2
G.E.	4	Duchenne	3	4	8	14	0.5
J.B.†	4	Duchenne	2	5	8	12	0.76
K.H.	3	Duchenne	8	4	CON	5	CON
M.C.‡	7	Duchenne	2	10	11	15	0.30
S.F.	4	Duchenne	9	6	8	13	0.50
S.W.	4	Duchenne	5	8	11	13	0.75
P.M.	23	Becker	2	9	9	14	0.1
M.P.	7	Becker	2	7	10	14	0.9
Controls							
A.D.*	15	spinal muscular atrophy (denervation)	2	NS	11	15	1.0
A.T.†	2	spinal muscular atrophy (denervation)	7	NS	8	12	0.73
E.H.‡	3	hereditary ataxia, normal muscle	2	NS	10	15	1.2
M.M.	2	normal muscle	2	NS	21	29	2.3
J.D.	4	nonspecific myopathy	4	NS	9	14	0.6
D.C.	7	fascioscapulohumeral dystrophy	5	NS	8	15	0.4
G.S.	6	nonspecific myopathy	4	NS	10	16	1.5
D.T.	2	nonspecific myopathy	4	NS	10	15	1.5
J.M.	7	Kugelberg-Welander	1	NS	15	22	0.01

CON, Contamination; DIV, days *in vitro*; CPK, creatine phosphokinase activity in μmol creatine per min per mg protein at 25°C ; NS, not seen.

*, †, ‡, Cultures run in identical conditions, that is, biopsy received the same day, same digestion, same media changes and so on.

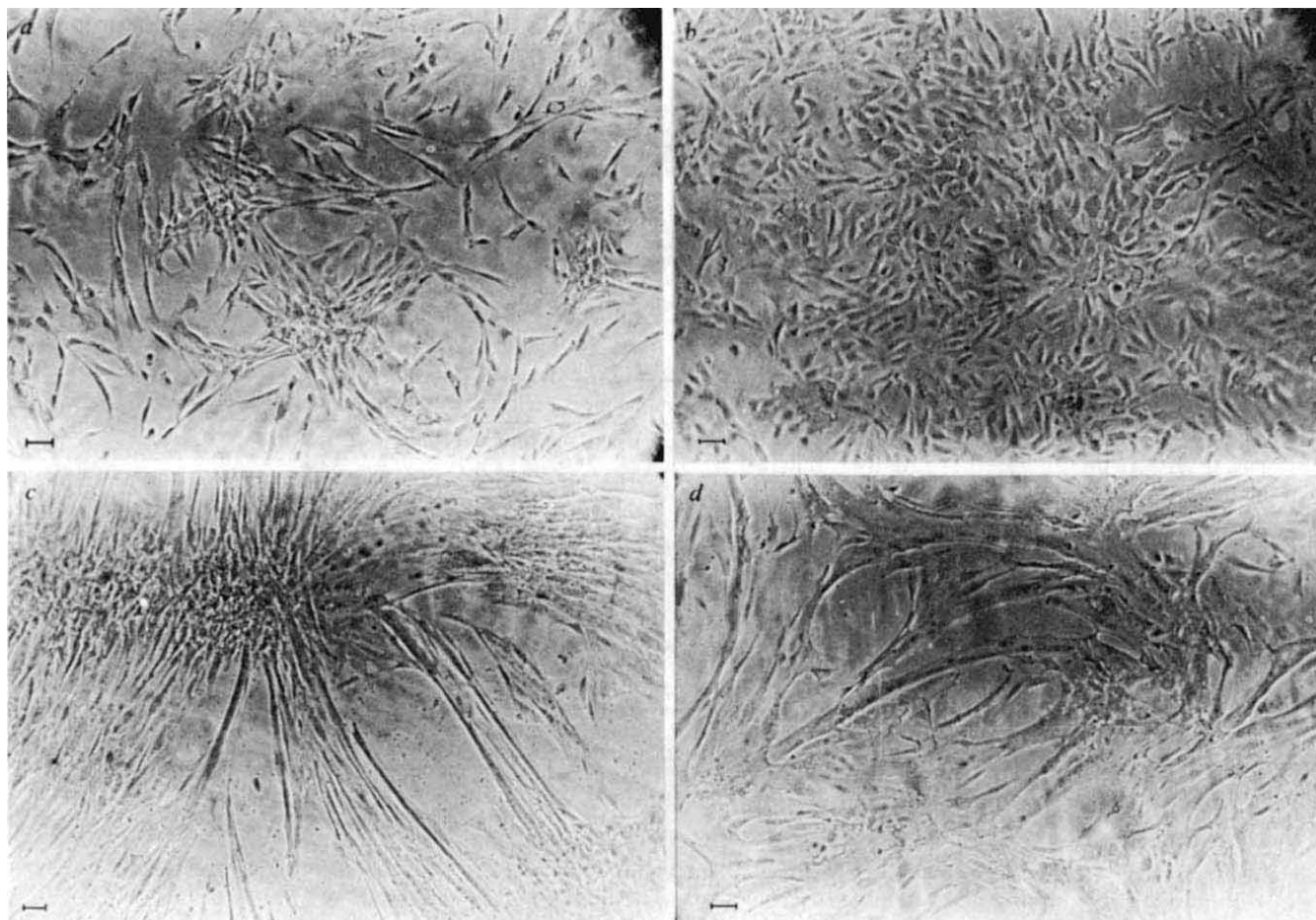


Fig. 1 General culture methods were as before⁸. Muscle was weighed and then submitted to four serial incubations with a mixture of 0.1% collagenase and 0.2% trypsin in a phosphate-buffered saline at 37 °C. The cells (6×10^4 per dish) (over 95% viable by dye exclusion) were plated on collagen-coated 35-mm Lux dishes in Dulbecco's Modified Eagle's Medium containing 10% (v/v) screened horse serum and 2% (v/v) detoxified chick embryo extract. *a*, Typical low power view of the hypertrophic clusters (see text for full description) from a Duchenne form of muscular dystrophy (JB). Culture was 6 d *in vitro*. *b*, Control culture for (*a*). Case of spinal muscular atrophy of similar age grown in identical culture conditions (A.T.): 6 d *in vitro*. *c*, Same case as (*a*) (Duchenne muscular dystrophy) but 11 d *in vitro* now showing abnormal muscle straps which span between hypertrophic clusters (see text for full description). *d*, Same case as (*b*) (control) but 11 d *in vitro*. Scale bars 50 μ m.

are found in the absence of any nerve cell input, at least this portion of the genetic defect is myogenic rather than neurogenic.

Clearly, further work is required to specify the precise biochemical alterations associated with these abnormalities in the initial cell appearance, their subsequent intercellular behaviour (and possibly cell membranes⁷) as well as their final degree of differentiation, including response to co-cultured nerve cells.

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A human leukaemic cell expressing hybrid membrane phenotypes

INDIVIDUAL surface markers differentiate B and T cells with varying degrees of certainty, and it is generally agreed that a panel of immunological markers is required to assign a particular cell to a category¹. In general, the ability to form spontaneous sheep red blood cell rosettes (E) and to react with anti-T-cell antisera are characteristics of T lymphocytes, whereas B lymphocytes are characterised by the presence of surface immunoglobulin (SmIg), C3 and Fc receptors and the presence of Ia-like antigens on

the cell membrane^{2,3}. However, in some instances lymphocytes are detected which exhibit combined B- and T-cell markers⁴. This has been particularly true in the case of human leukaemic cells, where a variety of unusual combinations of phenotypic markers has been described^{5,6}. In this communication, we report on a human leukaemic cell from a case of hairy cell leukaemia⁷⁻⁹ (HCL) with unusual combined B- and T-cell membrane characteristics.

In Table 1, data are presented on the surface marker characteristics of the unusual hairy cells (HCs) from peripheral blood and spleen when tested over a period of two months. Although the expression of these individual B and T phenotypic markers fluctuated over this time,

pathological cells of HCL enabled easy recognition of these cells both at light and electron microscope level in the various marker studies.

In the experiments to detect SmIg, a pre-incubation period of 1-2 h at 37 °C was routinely adopted, and the cells were observed to express SmIg of a single light chain type (*k*), of which IgD (*k*) was shown to persist after seven days in culture. The SmIg could also be removed by capping with anti-*k* chain serum. These points make it unlikely that SmIg can be attributed to cytophilic Ig or to autoantibody against the leukaemic cell.

To establish the nature of the E-rosette, a variety of blocking and inhibition experiments were carried out (Table

Table 1 Cell surface marker studies* on isolated mononuclear cells from peripheral blood and spleen

Source of mononuclear cells	Date	E	Anti-T	Percentage of cells rosetting or staining						Fc (μ)	Fc (γ)	P29/34
				μ	δ	γ	α	<i>k</i>	λ			
Peripheral blood	26.1.77	76	—†	5	18	5	—	19	3	22	—	—
	12.2.77	79	75	11	44	10	—	52	4	—	65	—
	26.2.77	52	70	77	37	72	0	70	16	66	78	79
Spleen	12.3.77	84	—	—	33	—	—	34	—	—	47	—
	12.1.77	73	—	5	82	—	0	78	0	—	96	90

*Peripheral blood (PB) mononuclear cells were separated by Ficoll-Hypaque centrifugation; a splenic cell suspension was prepared by forcing pieces of fresh spleen through a wire mesh and the mononuclear cells isolated using a Ficoll-Hypaque mixture. E-rosettes were performed by the method of Kaplan and Clarke¹⁹; the specific rabbit anti-T serum²⁰ (a gift from Dr G. Janossy) was employed using an indirect fluorescent method; SmIg was detected either by the rosette method of Ling *et al.*²¹ (purified sheep antisera a gift from Dr N. Ling) or by direct immunofluorescence (rabbit antisera a gift from Dr J. Smith); the Fc(μ) receptor was detected using rabbit IgM by the rosette method of Burns *et al.*⁹; the Fc(γ) receptor was detected using rabbit IgG by a rosette method; rabbit antiserum to purified human P29/34 (ref. 21) (a gift from Dr T. Springer) was used in an indirect fluorescent method (positive controls performed with B-cell leukaemias and negative controls with a T-cell line and a case of T-cell Sezary syndrome).

†Not done.

a significant overlap was found in the values obtained when the T cell markers (E-rosetting and anti-T cell serum) and the more conventional B cell markers (SmIg, Fc(γ) and the Ia-like protein complex P29/34) were compared. The summation of these B- and T-cell markers greatly exceeded 100%. This contrasts with the only reported case of a biclonal T- and B-cell leukaemia, in which the two clones independently expressed B and T membrane phenotypes¹⁰. In addition to the data presented in Table 1, the cells were found to be negative for the C3b receptor⁴ and the EB virus nuclear antigen (EBNA)¹¹, but did form spontaneous rosettes with mouse erythrocytes, a marker for a subpopulation of B cells¹² (1-9% with peripheral blood cells and 47% with splenic cells). In the spleen cell preparation, which was shown to contain >95% HCs, 95% of the cells were stained for intracytoplasmic IgM, IgG and *k* chain, of which 25% formed an intense patchy staining with FITC-labelled anti-IgG and anti-IgM. The finding of a receptor for IgM (Table 1) on these cells is not surprising, since this receptor is present both on a subpopulation of normal T lymphocytes¹³ and on the pathological cells of some B-cell leukaemias¹⁴ including HCL (ref. 9). The distinctive hairy appearance of the

2). Whereas E-rosette formation was blocked by the anti-T-cell serum (although this antiserum did not interfere with the detection of SmIg) it was not blocked by the anti-P29/34 serum. Furthermore, a reduction in E-rosette formation by HCs, similar to that seen with normal T lymphocyte controls, was observed when rosette formation was carried out at 4 °C, with and without centrifugation, and in the presence of sodium azide or cytochalasin B. Thus, like E-rosette formation by normal T lymphocytes¹⁵, this E-rosette formation was energy dependent and required an intact microfilament system. In addition, E-rosette formation was not markedly blocked either by group A substance from human saliva (which would block antibody activity against a heterophile antigen) or by treatment with anti-Ig sera. In the latter case, a SmIg receptor for sheep erythrocytes was further excluded by removing SmIg in a 'capping' process. In conditions in which no *k* chain was detected on the cell, E-rosette formation still occurred and, in some instances, the number of leukaemic cells rosetting with erythrocytes rose significantly.

HCL, a chronic leukaemia related to chronic lymphocytic leukaemia, is now considered to be a B-cell neoplasm^{7,8} but the cells have been reported as possessing a

Table 2 The effect on E-rosette formation of blocking with various substances and of capping of SmIg

Type of experiment	Percentage sheep RBC (E) rosette formation*							Sodium azide (0.1M)	Cytochalasin B (20 μg ml ⁻¹)
	Control	Anti-μ†	Anti-δ†	Anti-γ†	Anti-κ†	Anti-T‡	Anti-P29/34		
Blocking	52	53	36	52	59	0	59	—	—
	79	—	77	—	72	4	—	18	54
	52	69	66	82	65	—	—	—	—
Capping§	77	77	65	—	66	—	—	—	—

*Patient's leucocytes did not form spontaneous rosettes with a variety of other heterologous RBCs including ox, rabbit, guinea pig and rat.

†Anti-SmIg sera used at a concentration which totally inhibited SmIg-anti-SmIg rosette formation²¹.

‡This anti-T-cell serum²⁰ did not affect SmIg-anti-SmIg rosette formation.

§SmIg shown to have capped with each of the antisera by direct immunofluorescence and by failure to form SmIg-anti-SmIg rosettes.

number of monocytoid features such as adherence to glass and phagocytic ability⁷⁻⁹. Furthermore, the pathological cells of most cases of HCL are of indeterminate maturity since they show such features of immaturity as poorly condensed peripheral nuclear chromatin⁹ and the absence of a receptor for cholera toxin¹⁶. The nature of the disease, however, and the capacity to produce Ig for secretion suggests involvement of a mature cell type. It may not be surprising, therefore, that a cell of mixed B and T phenotype should appear in a leukaemia which is unusual in so many other ways, and it may indicate that the neoplastic transformation occurs at a point close to the divergence of B and T cells from the common haemopoietic stem cell. Alternatively, it may be that the genes controlling the expression of receptors for T-cell antigen have become derepressed by the neoplastic process acting on the HCs although derepression by EB virus is less likely due to the absence of EB nuclear antigen in these cells.

The argument of gene derepression has been proposed as the mechanism in the only previously described case of a human leukaemic cell, from a case of lymphosarcoma cell leukaemia, which possessed both SmIg and T-cell features¹⁷. Although there are some reports of leukaemic cells both forming E-rosettes and possessing receptors for Fc or C3, (refs 5, 6), there is evidence that subpopulations of normal lymphocytes also possess these marker combinations⁴ and such leukaemias may be an amplification of these minor subpopulations. No normal lymphocyte population bearing the marker characteristics of the leukaemic cells in this report has yet been described in man, but such cells may exist in the mouse¹⁸.

In conclusion, a case of HCL is described in which the pathological cells were shown to be E⁺, anti-T⁺, Fc(μ)⁺, SmIg⁺, cytoplasmic Ig⁺, P29/34⁺, Fc(γ)⁺, mouse erythrocyte rosette⁺, C3b⁻ and EBNA⁻. This observation emphasises the importance of using a large panel of immunological markers when studying membrane marker characteristics on haemic cells from both the normal and leukaemic situation. Indeed, cases of this kind could reveal further information on the nature of the leukaemic transformation and the study of such leukaemic cells may well lead to the identification of subpopulations of normal haemic cells.

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Epidermal Langerhans cells bear Fc and C3 receptors

THE mammalian epidermis contains a system of dendritic cells, eponymously referred to as Langerhans cells, whose functions are obscure. It is, however, accepted that Langerhans cells are of mesenchymal origin and since they are known also to occur in the dermis, lymph nodes, thymus, and other organs, the concept of Langerhans cells has widened to encompass a cellular system populating the connective tissues and certain stratified squamous epithelia of mammals¹. Among the many hypotheses about their biological significance they have been considered to represent immunocompetent cells or cells which might capture antigenic materials², but there is insufficient evidence for this. Lymphocytes have been observed to be closely associated with Langerhans cells in delayed type hypersensitivity reactions to dinitrochloro-benzene in guinea pigs³; and when guinea pigs passively sensitised with lymphocytes from ferritin-hypersensitive donors, were challenged with ferritin, ferritin-containing Langerhans cells were observed in the draining lymph nodes of the challenged animals⁴. Metals and certain amines have an affinity for Langerhans cells *in vitro*⁵, but since they can phagocytose ferritin and other substances without previous sensitisation¹, doubt remains whether they are immunologically engaged in the uptake and transport of potentially antigenic material. If Langerhans cells are indeed involved in immunological reactions, they should bear some of the surface markers characteristics of cells with known immunological functions, and we have now shown that they carry receptors for Fc and C3.

Epidermis of human volunteers was separated from the dermis, *in vivo*, using a suction blister device⁶, washed in Medium 199 (Flow Laboratories, Irvine, UK), and dissociated, *in vitro*, with 0.5% trypsin (Flow Laboratories, Irvine, UK) in phosphate-buffered saline (pH 7.2) for 30 min at 37°C. Epidermal cell suspensions were prepared by teasing and pipetting the epidermal specimens gently in Medium 199 supplemented with 50 mg ml⁻¹ gentamycin and 20% foetal bovine serum. After a double rinse and resuspension, the viability of the epidermal cells, checked by Trypan blue exclusion, ranged from 92 to 96%. The epidermal cells were then incubated with indicator reagents for Fc and C3 receptors, according to rosette assays described previously⁷: equal amounts of the epidermal cell suspension (5-10 × 10⁶ cells per ml) and of a 1% suspension of either IgG coated bovine red blood cells (EA-IgG) or IgM and complement-coated bovine red blood cells (EAC) were mixed, centrifuged and then incubated at 0°C for 1 h. Any cell binding three or more erythrocytes was scored as positive, a minimum of 200 cells being counted.

Multiple experiments consistently showed that 3-4% of the cells in the epidermal cell suspensions formed rosettes with the EA-IgG and the EAC reagent. Rosetting was not seen in the controls, which included incubation of epidermal cells with untreated bovine red blood cells (BRBC); BRBC coated with IgM antibody (EA-IgM); EA-IgM incubated with heat-inactivated or EDTA-inactivated fresh mouse serum; in the case of EA-IgG, epidermal cells pre-incubated with 10 mg ml⁻¹ heat aggregated human IgG or reacted with BRBC, coated with Fab₂ fragments of anti-BRBC IgG antibody. For this purpose the IgG anti-BRBC antibodies were pepsin digested according

to Nisonoff *et al.*⁸ and passed over a Protein A column (Protein A Sepharose CL 4B, Pharmacia, Uppsala, Sweden) to remove possible residual intact IgG molecules. BRBC coated with this Fab₂ antibody preparation (adjusted to the protein concentration of the original total IgG preparation) displayed no reactivity with either mononuclear blood cells or cells from epidermal cell suspensions.

Since the demonstrations of EAC rosettes after trypsin treatment contrasts with reports that EAC receptors on leukocytes are trypsin sensitive⁹, the influence of our trypsinisation procedure was checked on peripheral mononuclear cells. Whereas a 0.005% concentration of trypsin destroyed the C3 receptors on lymphocytes, EAC rosette formation with latex-ingesting monocytes was still detectable after treatment with 0.5% trypsin. Thus, the trypsin sensitivity of EAC rosette-forming cells from epidermal cell suspensions corresponded to that of latex-ingesting monocytes from the peripheral blood.

To determine the nature of the subpopulation of epidermal cells showing EA-IgG and EAC rosette formation, smears were prepared from cell suspensions treated with EA-IgG and EAC and incubated according to a modified¹ Wachstein and Meisel cytochemical technique¹⁰ for the demonstration of nucleosidetriphosphatase (NTP). This enzyme is, in the conditions used, a cytochemical marker for Langerhans cells¹. All rosette-forming cells of the epidermal cell suspensions were reactive for NTP, whereas the non-rosetting cells failed to show enzymatic activity. In addition, epidermal cells pelleted after exposure to the EA-IgG reagent were processed for electron-microscopy, either directly or after pre-incubation for NTP cytochemistry. At the ultrastructural level, all rosette-forming cells exhibited NTP activity, whereas non-rosetting cells did not; rosetting cells exhibited the classical ultrastructural morphology of LC, including the typical LC cell granules (Fig. 1), whereas non-rosetting cells showed morphological features of either keratinocytes (keratinising epidermal cells) or melanocytes. These results show that the subpopulation of epidermal cells which have the receptor for Fc and C3 are Langerhans cells.

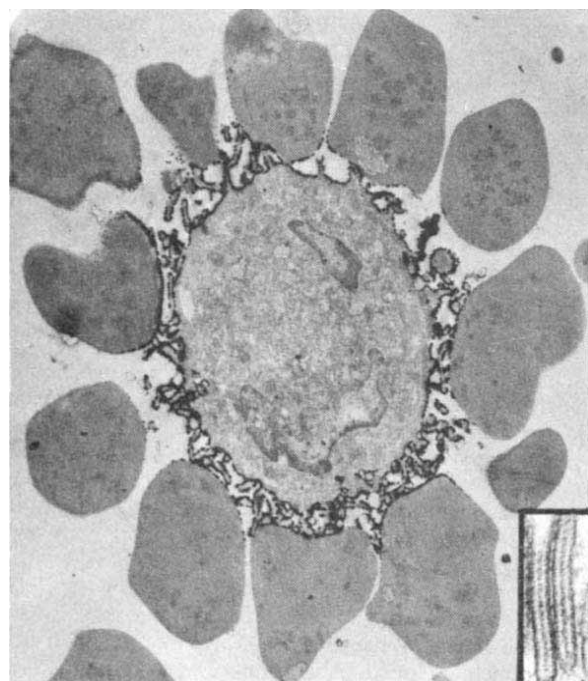


Fig. 1 Low-power electron micrograph of rosette from epidermal cell suspension treated with EA-IgG, incubated for the ultrastructural demonstration of NTP, and processed for electron microscopy. The rosetting, Fc receptor-bearing cell exhibits a folded nucleus, dendritic and villous surface projections, reaction product indicating NTP activity on its membrane and typical Langerhans cell granules in its cytoplasm (inset).

Fc and C3 receptors are known to occur on lymphocytes¹¹, on monocytes-macrophages histiocytes¹², and, as recently shown, on isolated hepatocytes¹³. Since Langerhans cells bear neither morphological nor cytochemical characteristics of lymphocytes¹ and since preliminary studies in our laboratories indicate that they exhibit neither membrane immunoglobulins nor E receptors, they do not qualify as lymphocytes. On the other hand, their Fc and C3 receptors make them reasonably good candidates for inclusion in the monocyte-macrophage-histiocyte series of cells despite the fact that, in phagocytosis experiments, *in vivo*, they fail to exhibit greater phagocytic capacity than the other cells of the epidermis¹⁴; also, the morphological identity of Langerhans cells with certain histiocytic cells in histiocytosis X (refs 1, 15) a proliferative histiocytic disease in man, is a good morphological correlate to these immunological findings. Our findings thus provide evidence for previous suggestions^{2,4,5} that Langerhans cells perform macrophage-like functions.

The cells of the monocyte-macrophage histiocyte system are known to have an important role in immune reactions. They are responsible for the uptake, processing, and transfer of antigens to immunocompetent lymphocytes^{16,17}, and they are involved in T-B interactions¹⁸. The biological role of their Fc and C3 receptors is not entirely clarified, but they seem to mediate antibody-dependent cellular cytotoxicity¹⁹ and the phagocytosis of immune complexes, whereby the C3 receptors bind C3 sites of immune complexes, and the Fc receptors are responsible for the ingestion phase²⁰. The antibody-binding capacity of macrophages may thus regulate the inductive phase of the immune response since cell-bound antibody can mediate the presentation of antigen to T lymphocytes²¹.

It does not appear unreasonable to assume that LC by means of their immunological receptor sites, perform functions similar to those of the monocyte-macrophage-histiocyte system, both by initiating the immune response to external antigens and by facilitating an enhanced reaction in the secondary response. These functions could thus provide a reasonable explanation for the paradox that Langerhans cells, as mesenchymal cells, are located in the most peripheral, ectodermal tissue of the mammalian organism, in the epidermis. If our conclusions are correct Langerhans cells may represent the most peripheral outpost of the afferent limb of the immune system.

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Ia antigen expression on human epidermal Langerhans cells

It has been proposed that the mammalian epidermal Langerhans cell is an epidermal equivalent of the cells of the reticulo-endothelial system, which are involved in binding and presentation of allergens in lymph nodes and the spleen^{1,2}. The demonstration that these resident cells are capable of binding various allergens and migrate to the lymph vessels and local draining nodes, indicates a major function in the afferent arm of the primary immune response^{3,4} and has improved understanding of contact allergic hypersensitivity reactions. Much still remains to be discovered concerning the characteristics of the cell type, its reactivity with various antigens, and its relationship to other cells of the immune system. We show here that epidermal Langerhans cells react positively by immunofluorescence with antisera raised in rabbits against human B lymphoblastoid cell line membrane glycoproteins, which indicates the presence of Ia-like antigens on these dendritic cells.

The Ia antigens are surface glycoproteins associated with particular groups of immunologically active cells⁵⁻⁷. Although they have been detected in small quantities on T cells they seem to be associated primarily with B cells^{8,9}. Recent studies have, however, shown them to also be expressed on macrophages¹⁰, sperm, foetal liver cells and epidermal cells¹¹. This last group is intriguing, since the observation was made by means of cytotoxic assays. Thus, no morphological distinction was made between the various cell types present in the epidermis. The proposal that Langerhans cells are a kind of histiocyte and the evidence pointing to the presence of significant numbers uniformly distributed in the epidermis¹², suggested that they might express Ia antigen. Since the functional significance of Ia antigens is not yet clear, confirmation of this proposal might have important implications. Ia antigens have been shown to be involved in mixed lymphocyte reactions (MLR)^{13,14}, graft-versus-host (GVH) reactions¹⁵, skin graft rejection¹⁶ and a variety of other cooperative phenomena.

Human B-lymphocyte Ia antigens were isolated from B lymphoblastoid cell lines (Fig. 1). The final protein peak used for production of rabbit heteroantisera, had a molecular weight of 33,000 daltons. The heteroantiserum after absorption with a human T cell line (MOLT 4), was shown to react against B cells but not T cells or thymocytes. When incubated with lymphocytes in the MLR, the antiserum suppressed thymidine incorporation. In precipitin analysis in Ouchterlony plates, a rabbit antiserum to HL-B antigens (Dr H. Kunkel, Rockefeller University), showed immunological identity indicating a relationship to the Ia system.

Human skin obtained at surgery was frozen and 5- μ m sections were prepared for direct and indirect immunofluorescence (IF) with the rabbit anti-Ia IgG fraction. Table 1 illustrates results of the various IF tests performed.

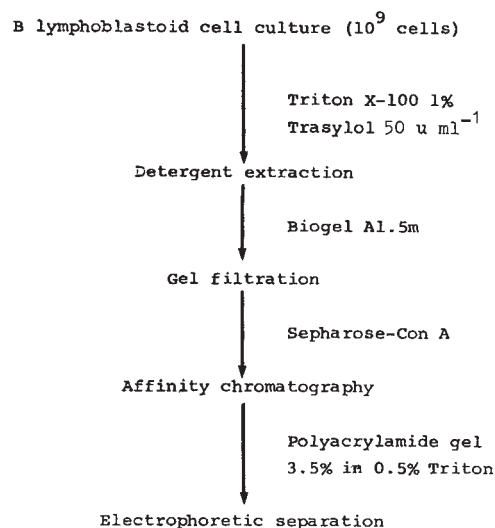


Fig. 1 Scheme of fractionation.

The findings can be summarised as follows: dendritic cells in the suprabasal zone of the human epidermis stain strongly at titres down to 1/1000 (Fig. 2). Specific immune blocking and pre-incubation with antigen confirmed the specificity of the reactions. The possibility that Fc receptors on Langerhans cells might nonspecifically bind immune reagents was tested using Fab' fragments of the rabbit anti-Ia antiserum. Positive staining was maintained using this reagent. Experiments to demonstrate either Fc receptors or receptors for C₃ using EA and EAC rosetting methods¹⁸ on frozen sections were negative. Other workers have failed to show binding of EA rosettes to cells in the epidermis, that might be interpreted as Langerhans cells (R. Edelson, personal communication).

In order to study further surface receptors on Langerhans cells a cell suspension of EDTA separated epidermis was prepared by 30-min incubation at 37 °C in 0.5% buffered trypsin. Epidermal cell suspensions were checked for viability, for morphology with haematoxylin and eosin, for ATPase staining and for the presence of Fc and C₃ receptors. EA (IgG) and EAC (IgM) rosetting methods were carried out on the cell suspensions with appropriate controls, that is, untreated SRBC and EA (IgM). With both EA and EAC methods between 2-3% of the cells formed rosettes of 5+ SRBCs. Rosetting was not seen in any of the controls. Thus cell suspensions of the epidermis prepared with trypsin demonstrate a small percentage capable of forming rosettes, indicating the presence of Fc and C₃ receptors on their surface.

Table 1 Fluorescent antibody staining results

Substrate	Procedure	Reaction	Comment
A Normal human skin	1 R-anti Ia*→GAR-FITC	+	Positive suprabasal dendritic epidermal cells
	2 Preimmune R-serum →GAR-FITC	—	
	3 Fab' of R-anti Ia→GAR-FITC	+	
	4 R-anti Ia→R-anti Ia FITC	—	Histiocytes and some lymphocytes in dermis positive
	5 R-anti Ia-FITC	+	
	6 GAR—FITC	—	
	7 Ia antigen+R-anti Ia→GAR-FITC	—	
	8 R-anti Ia→GAR-FITC	+	
	9 R-anti-Ia→GAR-FITC	—	
B Depigmentary conditions for example, vitiligo, halo nevus			Main basal dendritic epidermal cells positive
C Rat keratinising epithelia skin, oesophagus			No species cross-reactivity

*R-anti Ia, IgG fraction of rabbit anti-human Ia heteroantiserum.

GAR-FITC: IgG fraction goat anti-rabbit IgG conjugated to fluorescein isothiocyanate.

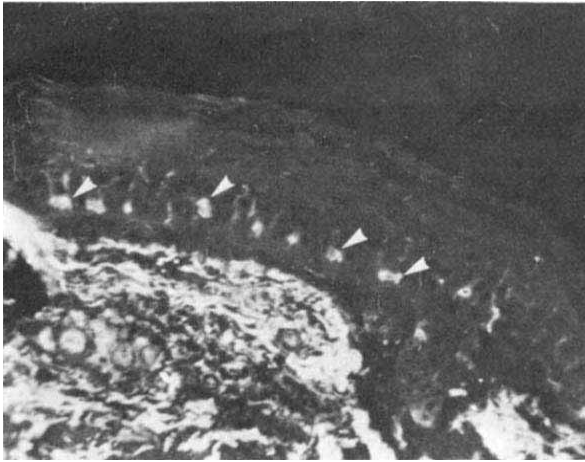


Fig. 2 Indirect immunofluorescence staining of normal human skin. Rabbit anti-human Ia (gG fraction) followed by goat anti-rabbit IgG-FITC positively stained suprabasal dendritic cells. $\times 238$ (arrows).

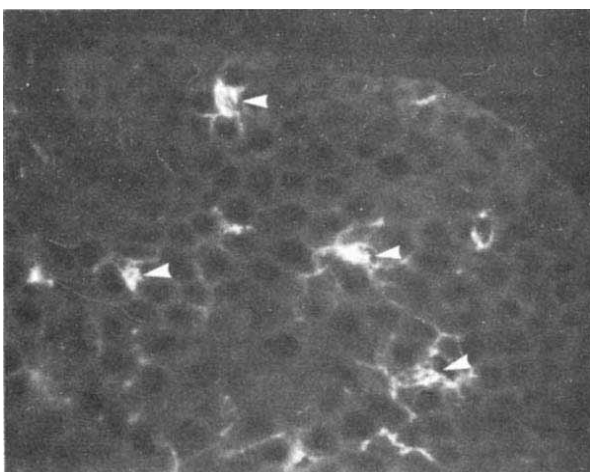
In addition, the same approximate percentage of cells show staining for ATPase and for Ia antigen as carried out by fluorescent microscopy. Simultaneous demonstration of the two reactions has not yet been achieved, but seems to be technically feasible.

As might be predicted from the information concerning distribution of Langerhans cells in leukodermatous^{19,20} conditions such as vitiligo and halo nevi, staining reactions were noted not only in the suprabasal location but also in the basal layer. Furthermore there was no cross-species reactivity since Langerhans cells in rodent keratinising epithelia did not stain.

These observations suggest that if the Langerhans cell is a histiocyte, it is atypical, since it does not seem from frozen sections, to express either Fc or C₃ receptors that are detectable by rosetting, nor is it very phagocytic²¹. Clearly the disparity in the detection of surface receptors on isolated cells as opposed to frozen sections can be explained either by the sensitivity of the methods or because of an unmasking effect of the trypsinisation. Whether the cells *in vivo* express these surface receptors requires further investigation. The specific staining with anti-Ia antisera should provide a tool to assist in the isolation of the Langerhans cell.

To confirm these findings ultrastructurally, we are using Fab' fragments of the anti-Ia antiserum labelled with ferritin in electron microscopic studies. Preliminary results demonstrate a weak but specific staining of the membranes

Fig. 3 Indirect immunofluorescence staining of a tangential section of normal human skin (as in Fig. 2). High magnification demonstration of dendritic cells. $\times 680$.



of epidermal Langerhans cells. In the same studies dermal histiocytes and some lymphocytes also show specific staining. Clearly the present identification of positively stained cells in the epidermis as being Langerhans cells relies heavily on the established suprabasal localisation in normal tissue and is supported by the changes in distribution seen in leukodermatous skin. The cells as shown in a tangential section are clearly dendritic (Fig. 3). ATPase-stained tissue sections show an identical distribution pattern.

These observations provide interesting avenues for research on the Langerhans cell itself, as well as confirming that they express surface antigens that have so far been associated with immunologically active cells. They also have a role with such cells in the rejection of skin grafts.

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Epidermal Langerhans cells express Ia antigens

THE major histocompatibility complex (MHC) of mouse and man controls the expression of several cell surface antigens¹. The classical transplantation antigens are present on most if not all adult, nucleated cells. However, molecules controlled by the immune response region of the MHC display a much more restricted tissue distribution. The immune response-associated antigens (the Ia antigens in the mouse and HLA-D antigens in man) seem to be integral parts of the plasma membrane of B and T lymphocytes, macrophages, spermatozoa, and epidermal cells². The physiological role of the Ia antigens is far from understood but their participation in various immunobiological events is well documented (for a review, see ref. 3). In addition to their role as triggers in the mixed leukocyte reaction⁴, the Ia antigens are required for collaboration between T and B lymphocytes⁵ and between macrophages and T cells⁶. Since all known functions of the Ia antigens pertain to the immune system it seemed that they should be expressed on an epidermal cell population involved in immune reactions. We therefore set out to investigate which of the three cell types of epidermis, keratinocytes, melanocytes, and Langerhans cells, expresses the Ia antigens, and from the results of fluorescent antibody staining we conclude that it is the Langerhans cells.

Epidermal cells remove anti-Ia antigen antibodies from alloantisera² but no data are available as to the biochemical nature of the Ia antigen-reacting molecules in epidermis. To reveal the structure of these molecules biopsies of skin were obtained from patients undergoing surgery for mammary hyperplasia. The epidermis was separated mechanically from underlying dermis after heating the biopsies to 52 °C. The biopsies were treated by homogenisation on 0.02 M Tris-HCl

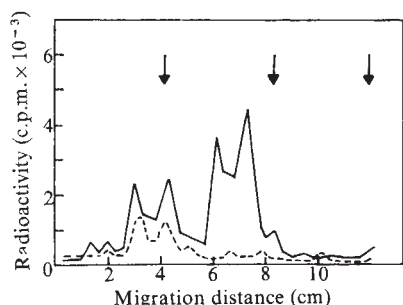


Fig. 1 SDS-polyacrylamide gel electrophoresis in reducing conditions of Triton X-100-solubilised, ¹²⁵I-labelled epidermal glycoproteins reactive with a rabbit antiserum raised against highly purified HLA-D antigens (—). Normal rabbit serum served as the control (---). The details of the experimental procedure have been outlined elsewhere⁷. The arrows denote the migration positions (from left to right) of marker heavy and light chains of human IgG and the marker dye bromophenol blue.

buffer, pH 8.0, containing 0.15 M NaCl and 1% Triton X-100. Insoluble material was removed by centrifugation at 105,000g for 60 min and glycoproteins, present in the supernatant, were isolated on an affinity chromatography column containing covalently-bound *Lens culinaris* haemagglutinin⁷. Purified glycoproteins were labelled with ¹²⁵I and Ia antigen-reacting material was isolated by indirect immunoprecipitation using a specific rabbit anti-HLA-D antigen serum⁸. The immune complexes were dissolved and separated by SDS-polyacrylamide gel electrophoresis, as previously described⁷. Figure 1 shows that the anti-HLA-D antigen serum preferentially precipitated two types of polypeptide chains with the apparent molecular weights 28,000 and 34,000. This two-chain pattern is indistinguishable from that obtained when the same antiserum was used to precipitate molecules obtained from leukaemic B lymphocytes. However, two additional components with the apparent molecular weights 70,000 and 50,000, respectively, were also visualised. Since the same polypeptide chains were also present in precipitates generated by normal rabbit serum,

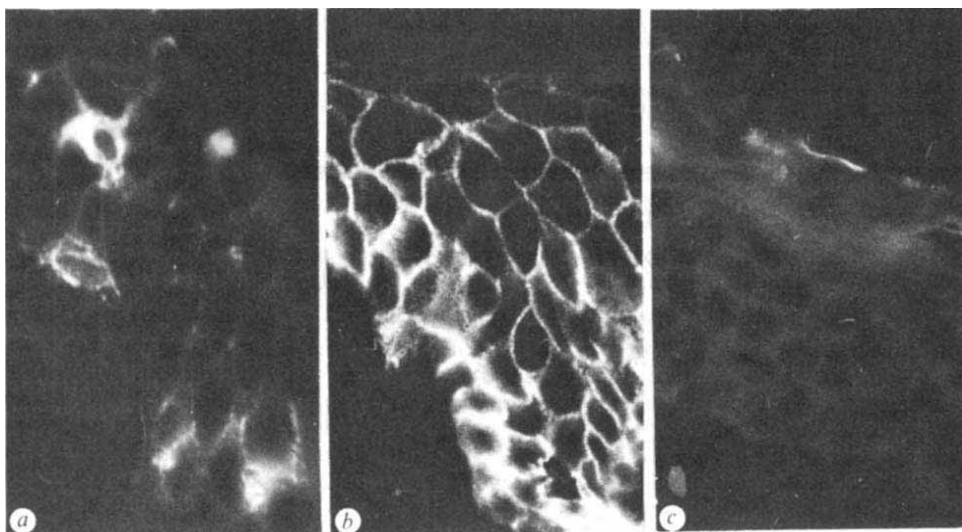
it seems reasonable to conclude that they represent nonspecifically-precipitated contaminants. Thus, the result is consistent with the view that HLA-D antigens of epidermis have the same overall structure as their counterparts on B lymphocytes.

The type of cell in epidermis which expresses HLA-D antigens was examined with use of the IgG-fraction of a rabbit anti-HLA-D antigen serum. Biopsies of human skin were obtained from the wrist of healthy volunteers. Thin sections were prepared from the biopsies and the presence of HLA-D antigens was assessed by an indirect immunofluorescence technique, as described in the legend of Fig. 2. Antibodies against HLA-D antigens only stained a few cells in the epidermis. Figure 2 shows that the stained cells were confined to the suprabasal part of epidermis and that they displayed dendritic processes. The appearance of the stained cells as well as their localisation in the epidermis suggest that they are not keratinocytes. To demonstrate that the latter cell type does react with antibodies, thin sections of skin biopsies were stained with antibodies against β_2 -microglobulin⁹. Since β_2 -microglobulin represents the common subunit of the HLA-A, -B, and -C antigens¹, which are known to be present on keratinocytes, the antibodies should stain all cells in epidermis. Figure 2 demonstrates that antibodies against β_2 -microglobulin gave a bright intercellular staining of all cells in the epidermis but dermis was not stained. The specificity of the antibody binding was assessed by replacing the antisera by normal rabbit gamma globulin. Figure 2 shows that normal rabbit gamma globulin did not stain any cells in the epidermis.

Since the above data clearly demonstrate that the HLA-D antigen-bearing cells are not keratinocytes an experiment was performed to investigate whether the antigens were present on melanocytes or Langerhans cells. Advantage was taken of the fact that the epidermis of albino guinea pigs virtually lacks melanocytes¹⁰. We have previously shown that the antiserum cross reacts extensively with murine⁸ and guinea pig Ia antigens (L.K., unpublished observation). Despite the absence of melanocytes in the guinea pig epidermis staining of the HLA-D antigens gave a similar pattern to that observed for human epidermis. This demonstrates that the melanocytes are not responsible for the presence of Ia antigens in the epidermis. Thus, the observations that keratinocytes and melanocytes do not express HLA-D antigens and the dendritic appearance of the HLA-D-bearing cells strongly suggest that it is the Langerhans cells which express these antigens.

The function of the Langerhans cells is far from understood but they may represent macrophage-like scavengers with poor phagocytic activity¹¹. The recent observations that the Langer-

Fig. 2 Immunofluorescence of thin sections of skin biopsies after indirect staining with antibodies against HLA-D antigens (a) and β_2 -microglobulin (b). In c normal rabbit gammaglobulin replaced the antibodies. Sections of 6 μ m were prepared from skin biopsies. The air-dried sections were separately incubated with 50- μ l portions of the IgG fractions from the antisera and normal rabbit serum. The IgG fractions were diluted in phosphate-buffered saline (pH 7.2) containing 4% of bovine serum albumin. The concentration of IgG was varied from 1 to 0.1 mg ml⁻¹. The antibodies were allowed to react with the skin sections for 30 min at room temperature in a humid atmosphere, then washed extensively in the phosphate buffer, and allowed to react with specific fluorescein-isothiocyanate-conjugated goat anti-human IgG at a working concentration of about 0.1 mg ml⁻¹. After an incubation period of 30 min and several washes the sections were mounted in 10% glycerol and examined in a



Leitz Orthoplan fluorescence microscope with incident light and blue narrow band activation illuminated with a Philips $\times$ BO 75 lamp.

hans cells selectively take up antigens which are known to cause contact dermatitis^{12,13} and the present finding that these cells express Ia antigens are in keeping with an immune function for the Langerhans cells. It is conceivable that the role of the Langerhans cells in contact dermatitis may be related to the following observations. It seems likely that T killer cells participate in contact dermatitis¹³ but the generation of T killer cells is generally dependent on allogeneic differences involving the classical transplantation antigens as well as the Ia antigens¹⁴. However, chemically modified syngeneic cells may also give rise to the production of T killer cells that are directed against the individual's own transplantation antigens as well as to the foreign antigen¹⁵. The stimulation by the foreign antigen of the precursors of the T killer cells seems to require that the antigen is presented by macrophages expressing syngeneic Ia antigens¹⁶. Contact dermatitis may thus involve binding of the allergen to the Langerhans cells, which by their expression of Ia antigens may trigger antigen-specific T lymphocytes to become killer cells. These killer cells will subsequently react with any cell which expresses the syngeneic transplantation antigen together with the allergen. Since in epidermis Langerhans cells preferentially bind those antigens which are known to cause contact dermatitis the Langerhans cells should be the prime target cells. Interestingly, in contact dermatitis the number of Langerhans cells is reduced and the apposition of mononuclear cells to Langerhans cells has been observed¹³.

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Cytotoxicity against tumour-associated antigens not H-2 restricted

CYTOTOXIC thymus-derived lymphocytes (T cells) can be generated both *in vivo* and *in vitro* against many different types of cell surface antigen (reviewed in ref. 1). There have been several reports that when mice were injected with non-oncogenic viruses^{2–4} or when spleen lymphocytes were sensitised *in vitro* against chemically modified syngeneic cells⁵, the resultant cytotoxicity was directed only against target cells which shared with the sensitising and/or effector cells in the H-2K or H-2D region of the major histocompatibility complex (MHC). Similar observations have been made with several experimental tumour systems^{6–10}. This indicates that the interaction between immune cytotoxic T lymphocytes and target cells is H-2 restricted and compatibility at the H-2K or H-2D regions of

the MHC is essential. But we now have data which indicate that identity at the H-2 complex (or at subregions within this complex) is not required for cytotoxic reactivity between T lymphocytes from mice immunised against murine sarcoma virus (MSV) and tumour-associated antigens on target cells. Spleen cells from immune animals could lyse either syngeneic or allogeneic tumour targets carrying the appropriate tumour-associated antigens. Thus it seems that the antigenic moiety against which the MSV-immune T cell is directed is not exclusively a modification of the H-2 antigen. Furthermore, the magnitude of the cytotoxicity against allogeneic target cells depended on the strain of animal used for immunisation.

BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, 8–12 weeks old, were injected intramuscularly in the leg with a regressor strain of Moloney-MSV. Tumours appeared 7–9 d later, reached maximum size at about 14 d, and then regressed. Spleen cells were collected from the mice 12–14 d after virus inoculation—the peak of cell-mediated cytotoxicity as measured by the ⁵¹Cr-release cytotoxicity assay^{11,12}—and tested for their cytolytic activity in a 16–18-h ⁵¹Cr release assay. The target cells were Rauscher (RLV) or Moloney (MLV) virus-induced lymphomas or chemically induced lymphomas of C57BL/6, BALB/c, A/J or DBA/2 mice (Table 1). These chemically-induced lymphomas were not susceptible to lysis by syngeneic T cells from MSV-immune mice and served as specificity controls.

Spleen cells from both C57BL/6 and BALB/c MSV-immunised mice exhibited strong cytotoxic reactivity against MLV- or RLV-induced syngeneic tumour targets as shown by the representative experiments in Tables 2 and 3. This agrees with previous reports^{11,12}. But various levels of cytotoxicity were also observed against allogeneic MLV or RLV tumour target cells, with the strength of the response dependent on the strain of mouse from which effector cells were derived. C57BL/6

Table 1 Description of lymphoma tumour lines

	Strain of origin	H-2 type	Inducing agent
RBL-5	C57BL/6	H-2 ^b	RLV
MBL-2	C57BL/6	H-2 ^b	MLV
EL-4 (G-)	C57BL/6	H-2 ^b	Benzpyrene
YC8	BALB/c	H-2 ^d	MLV
LSTRA	BALB/c	H-2 ^d	MLV
MCDV-10	BALB/c	H-2 ^d	RLV
L1210	DBA/2	H-2 ^d	Methylcholanthrene
YAC	A/J	H-2 ^a	MLV

spleen cells showed very strong activity against tumour-associated antigens on allogeneic tumour cells. On the other hand, BALB/c effector cells were not as efficient at lysing C57BL/6 tumour targets, for although significant levels of cytotoxicity against allogeneic targets were observed in some cases (Table 2 experiment 1b, MBL-2 and experiment 2b, RBL-5), reactivity was mostly very low. Cytolysis was specific and not directed against alloantigens (Table 3). MSV-immune B6 effector cells were cytotoxic against syngeneic (H-2^b) and allogeneic (H-2^d or H-2^a) tumour targets but did not lyse allogeneic phytohaemagglutinin (PHA) blasts. Furthermore, there was no lysis against H-2 identical tumour cells which did not carry the appropriate antigen (Table 2 experiments 1a and 2b) even though these targets could be lysed by alloimmune cytotoxic effector cells (data not shown). When the effector cells were treated with anti-Thy 1.2 antibody plus complement, they could not mediate cytotoxicity, thus confirming the T-cell nature of this immune function (Table 4).

Direct cytotoxic reactivity against tumour-associated antigens on allogeneic tumour cells has not been described before. Herberman *et al.*¹³, using a 4-h ⁵¹Cr release cytotoxicity assay, saw no cross reactivity against tumour target cells of another strain. But the more sensitive cytotoxicity inhibition assay showed several of the allogeneic tissue culture lines to have

Table 2 Cytotoxic reactivity against tumour-associated antigens on syngeneic and allogeneic tumour cells

MSV immune spleen cells from	RBL-5 (H-2 ^b)	MBL-2 (H-2 ^b)	EL-4(G-) (H-2 ^b)	Target cells* YC8 (H-2 ^d)	LSTRA (H-2 ^d)	MCDV-10 (H-2 ^d)	L1210 (H-2 ^d)
Experiment 1							
(a) C57BL/6 (H-2 ^b)	35.9 (0.6)+	†37.0 (1.4)+	-1.1 (0.9)	36.4 (1.2)+	18.2 (1.5)+		
(b) BALB/c (H-2 ^a)	0.8 (0.4)	5.2 (1.4)+		19.6 (1.0)+	20.3 (1.1)+		
(c) BALB/c (H-2 ^d)	-2.2 (1.5)	2.2 (1.0)		17.8 (1.3)+	15.3 (1.6)+		
(d) BALB/c (H-2 ^d)	-2.4 (1.3)	0.3 (0.3)		39.3 (0.9)+	18.8 (1.3)+		
Experiment 2							
(a) C57BL/6 (H-2 ^b)	54.4 (1.2)+			29.7 (2.0)+		25.9 (0.7)+	
(b) BALB/c (H-2 ^a)	16.4 (1.4)+			27.2 (1.3)+		14.7 (1.5)+	
(c) BALB/c (H-2 ^d)	1.2 (0.9)			27.9 (1.3)+		22.9 (1.5)+	-1.7 (1.3)
(d) BALB/c (H-2 ^d)	0.2 (1.2)			32.8 (1.3)+		21.4 (0.8)+	

2.5×10^6 spleen cells from animals immunised 12–14 d earlier with MSV were mixed with 1.25×10^4 ^{51}Cr -labelled tumour cells in 35-mm Petri dishes and incubated for 16–18 h at 37 °C on a rocking platform. Supernatants were collected and processed as previously described¹⁸. Spontaneous ^{51}Cr release was usually 15–35% of the total unless otherwise indicated.

*Percentage specific release of ^{51}Cr ($\pm$ s.e.) from the tumour cells was calculated according to the formula

$$\frac{\text{c.p.m. supernatant experimental} - \text{c.p.m. supernatant control}}{\text{Total c.p.m. incorporated into cells}} \times 100$$

† $P < 0.01$ as calculated by Student's *t* test compared with control.

detectable levels of antigen^{13,14}. On the basis of these inhibition studies, it was concluded that the cytotoxic reactivity of spleen cells from MSV-injected C57BL/6 mice was directed against an endogenous C-type virus-associated antigen, MEV-SA-1, and even xenogeneic target cells infected with the appropriate virus were found to carry the antigen^{15,16}. In contrast, Gomard *et al.*⁹ examined the interrelationship between T-lymphocyte cytotoxicity and products of the H-2 complex and concluded that recognition of antigens on the surface of tumour cells by MSV immune T lymphocytes was determined by an H-2 region product. The results of an 18-h ^{51}Cr release assay, however, confirm our previous inhibition data and demonstrate that substantial levels of cytotoxic reactivity can occur against allogeneic tumour cells (especially when effector cells from C57BL/6 MSV-immune mice are used) even though reactivity is still higher and occurs earlier against syngeneic targets (our unpublished observations). Thus in this system we see H-2 preference but not H-2 restriction. Similar observations have been made in virus-infected or hapten-modified systems where some cytotoxic reactivity was also observed against allogeneic modified target cells^{17,18}.

A further point should be made regarding the specificity of the cytotoxic reactivity. As noted, the antigen recognised on syngeneic tumour cells by C56BL/6 MSV-immune cells is MEV-SA-1. We have not so far identified the antigenic specificity (or specificities) in addition to MEV-SA-1 which can be recognised by these mice on the allogeneic target cells nor have we identified the antigens recognised by BALB/c MSV-immune effector cells. Nevertheless, although the mice were injected with Moloney-MSV, the cytotoxic reactivity against either

syngeneic or allogeneic tumour targets did not seem (as suggested¹⁹) to be associated with a type-specific MLV-induced antigen, for reactivity was observed with each mouse strain against both syngeneic and allogeneic MLV- and RLV-induced lymphomas.

Our findings suggest several models for the control of the specificity of the interaction between tumour-associated antigen and T lymphocytes. First, the tumour-associated antigens induced by the oncogenic viruses in different strains of mice are similar or identical (therefore unrelated to H-2 products)

Table 4 Treatment of MSV-immune effector cells with anti-Thy 1.2 plus complement

C57BL/6 (H-2 ^b) MSV-immune spleen cells	Targets MBL-2 (H-2 ^b)	YC8 (H-2 ^d)
Untreated	33.8 (2.2)	11.3 (1.5)
Media control	34.4 (2.0)	12.5 (1.3)
Complement	34.0 (2.3)	11.2 (1.5)
Anti-Thy 1.2 plus complement	0 (2.3)	-0.9 (1.2)

Spleen cells from C57BL/6 mice immunised with MSV were treated twice according to the following protocol: 10^6 spleen cells were incubated with 0.6 ml of medium or anti-Thy 1.2 (final 1:2 dilution) for 30 min at 22 °C. After one wash the cells were treated with medium or with rabbit complement for 45 min at 37 °C. The cells then were washed twice with medium, resuspended to the same viable cell count, and then assayed for cytotoxicity in the 18-h ^{51}Cr release cytotoxicity assay against two different tumour targets.

and a receptor on the T lymphocyte for the H-2 of the sensitising cell enables the killer cells to interact more effectively with the target. Such a model would accord with the preference for the syngeneic targets expressed by the MSV effector cells. Second, there are several types of cell surface antigens induced by the virus, with each mouse strain, expressing unique antigens as well as common antigens; in addition, cytotoxic reactivity is stronger against the unique antigens. In such a case, there would have to be at least two subpopulations of effector T cells generated by MSV immunisation, one which reacts predominantly against the unique antigen on syngeneic cells and another which reacts with tumour-associated antigens with allogeneic cells. Third, immune response genes may be controlling the generation of the cell-mediated response and, in such a case, the animal may be able to recognise only some of the antigens induced by the virus. Therefore, although the antigens expressed on the tumour cells may have some common antigenic moieties, the response may be restricted by the ability of the host to recognise that determinant. We cannot distinguish whether one or more of these alternatives are operative in the MSV system.

Table 3 Specificity of the cytotoxic reactivity by MSV-immune lymphocytes in the 18-h ^{51}Cr release assay

		Effector cells*		
Target cells H-2 type		C57BL/6 (H-2 ^b) MSV immune	C57BL/6 (H-2 ^b) anti-P815 (H-2 ^d)	Baseline† release
RBL-5	H-2 ^b	28.7 (2.1)+	0	23.3
MBL-2	H-2 ^b	24.8 (0.7)+	0	43.6
MCDV-12	H-2 ^d	20.0 (0.7)+	41.3 (0.8)+	23.2
YAC	H-2 ^a	21.2 (1.1)+	41.4 (3.0)+	21.5
PHA blasts				
BALB/c	H-2 ^d	2.5 (1.4)	13.8 (1.7)+	40.7
DBA/2	H-2 ^d	0	18.0 (3.7)+	47.9

*See footnotes for Table 2.

†Baseline control values were determined using normal thymocytes or non-reactive lymphocytes that gave release values which were below or not significantly different from the thymocyte control.

Similar to our demonstration of reactivity *in vitro* of immune lymphocytes against syngeneic and allogeneic tumour targets, *in vivo* immunisation procedures with these tumour lines have shown that by immunising with an allogeneic BALB/c lymphoma (LSTRA) C57BL/6 mice could be protected from challenge with a syngeneic tumour (MBL-2)²⁰. T lymphocytes have a major *in vivo* role in protection against tumour challenge^{21,22} and therefore assuming that the activity detected *in vitro* accurately reflected the events taking place *in vivo*, the cytotoxicity we have observed against BALB/c tumour cells by MSV-immune C57BL/6 lymphocytes might have been anticipated.

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In previous studies on induction of β -adrenergic receptors by short-chain fatty acids we noted that while 5 mM pentanoic acid was as effective as 5 mM butyric acid for induction of β -receptors as measured by binding of ^3H -alprenolol, cyclic AMP production in response to isoproterenol stimulation was only increased about one-third as much by pentanoate as by butyrate⁷. This observation suggested that the specific receptor could be induced in a form which is functionally 'uncoupled' from adenylate cyclase. The total cellular activity of adenylate cyclase did not increase with butyrate treatment; Table 1 shows basal, fluoride-stimulated, and cholera toxin-stimulated adenylate cyclase activities in homogenates of untreated and 5 mM butyrate-treated HeLa cells. The induced receptors apparently become functional by associating with pre-existing adenylate cyclase. As expected, *l*-isoproterenol-stimulated adenylate cyclase activity was higher in homogenates of 5 mM butyrate-treated cells. When induction of receptors was examined as a function of butyrate concentration, we found that while ^3H -alprenolol binding was fully induced by low concentrations of butyrate, 10-fold higher concentrations of the fatty acid were required for full expression of *l*-isoproterenol-stimulated cyclic AMP production, as shown in Fig. 1. In general, although higher concentrations of pentanoate were required for induction of binding, at 5 mM the same levels of activity were reached with either fatty acid; pentanoate, however, was far less effective than butyrate in eliciting catecholamine-stimulated cyclic AMP production, in agreement with our earlier observations (data not shown).

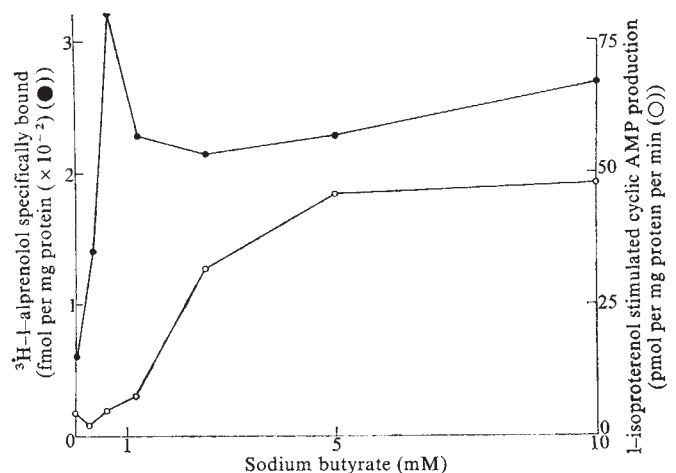


Fig. 1 Effects of various concentrations of butyrate on induction of β -adrenergic receptors and *l*-isoproterenol-stimulated cyclic AMP production. HeLa cells (Rhino; GIBCO) were grown as previously described¹⁹ in minimal essential medium with non-essential amino acids and 10% foetal calf serum. 150-mm plastic tissue culture dishes were seeded at $(3-4) \times 10^4$ cells cm^{-2} and incubated 24 h at 37 °C. The medium was replaced with fresh medium containing the indicated concentration of butyrate and the incubation continued for an additional 24 h. Dishes were washed with 10 ml phosphate-buffered saline containing 0.2% gelatin (PBS), cells collected by scraping in 10 ml PBS, and collected by centrifugation (3,000 r.p.m. for 4 min at 4 °C). Cells from one 150-mm dish were suspended for assay in 2 ml 0.9% NaCl, 50 mM Tris-HCl (pH 7.4) containing 5 mM theophylline and 9 mM 3-isobutyl-*l*-methylxanthine. Specific binding of ^3H -dihydroalprenolol and *l*-isoproterenol-stimulated cyclic AMP production were determined as previously described⁷.

Time course studies indicated that butyrate induction of ^3H -alprenolol binding was complete by 10 h with either 0.5 mM or 5 mM butyrate (ref. 7 for 5 mM, unpublished data for 0.5 mM butyrate); the lower concentration of butyrate induced about 80% of the binding activity induced by the higher concentration. But, 0.5 mM butyrate was far less effective than 5 mM butyrate in eliciting catecholamine-stimulated cyclic AMP production (Table 1). The differential effect of the two butyrate concentrations could not be attributed to slower induction by the lower concentration.

Relationship between β -adrenergic receptors and adenylate cyclase in HeLa cells

The physiological actions of many hormones and neurotransmitters are mediated through activation of adenylate cyclase (ATP pyrophosphate lyase [cyclising] EC 4.6.1.1) by specific cell surface receptors for each substance. The consequent increases in intracellular cyclic AMP lead to activation of protein kinases and a host of alterations in cell physiology, depending on the type of cell stimulated¹. Adrenergic receptors, responsive to β -hydroxylated catecholamines, were divided into α - and β -types by Ahlquist² using classical pharmacological techniques. The β -adrenergic effects of catecholamines occur through a receptor-mediated stimulation of adenylate cyclase activity³. Although β -adrenergic receptors have often been described as 'tightly coupled' to adenylate cyclase, recent evidence suggests that the receptor and the enzyme are physically discrete entities^{4,5}, probably the products of different genes⁶. We have found that HeLa cells contain β -adrenergic receptors and that the number of receptors per cell, but not the cellular content of adenylate cyclase, increases markedly during exposure of the cell to butyrate⁷. Here we present direct evidence that the HeLa cell β -adrenergic receptor can exist in a form either 'coupled' to or 'uncoupled' from adenylate cyclase. A model system for studies of receptor-adenylate cyclase interactions is described, and preliminary results of studies on the mechanism of coupling are reported.

Table 1 Cyclic AMP production in homogenates of HeLa cells after various treatments

Activity measured	Additions to medium		
	None	0.75 mM butyrate	5 mM butyrate
	(pmol cyclic AMP produced per min per mg protein)		
Basal*	8.2	9.2	11.4
NaF-stimulated*	135	152	147
Isoproterenol-stimulated*	8.8	22.4	50.6
Cholera toxin-stimulated†	70	ND‡	50

*HeLa cells were incubated 24 h in medium containing the indicated concentrations of butyrate, homogenates prepared, and cyclic AMP determined as previously described⁷.

†HeLa cells were incubated for 22 h in medium containing the indicated concentrations of butyrate, cholera toxin was added (4 µg ml⁻¹, 25 ml per dish) and the cells incubated an additional 2 h; homogenates were prepared and cyclic AMP measured as above. In this separate experiment the basal activity of adenylate cyclase (pmol cyclic AMP per min per mg protein) for untreated cells and 5 mM butyrate treated cells were 13.0 and 5.0 respectively.

‡ND, not determined.

An important aspect of the differential effect of the low (0.5 mM) compared with the high (5 mM) butyrate concentration is that it permits us to obtain cells with large numbers of non-functional receptors (Table 1) and, by manipulation of the tissue culture medium contents, to convert them to functional receptors; we will refer to experiments of this type as 'coupling' studies. Additional studies indicated that 0.75 mM butyrate was optimal for maximal differential between binding activity and catecholamine-stimulated cyclic AMP production. After β -receptors were induced by exposing cells to 0.75 mM butyrate, the time course was studied by replacing the initial medium with fresh medium containing 5 mM butyrate. As shown in Fig. 2, coupling begins after a brief lag and increases sharply during the next 6 h. This experimental design was used then to study the effects on coupling of certain inhibitors and medium components.

Table 2 summarises the results of several experiments in which the effects of various inhibitors or omission of certain medium components were examined separately on both the one-step induction of functional receptors and on the coupling of pre-induced receptors with adenylate cyclase. Cycloheximide, at a concentration which we have previously found to inhibit protein synthesis more than 90% in HeLa⁸, completely blocked induction of functional receptors when included with 5 mM butyrate during a 24-h incubation. Surprisingly, cycloheximide caused a 90% decrease in pre-induced hormone binding after 24 h in coupling experiments, perhaps suggesting a rapid turnover of receptor molecules. Catecholamine-stimulated cyclic AMP production was less severely suppressed which may indicate that coupling of surviving receptors to adenylate cyclase does not require protein synthesis. Whether or not RNA synthesis is necessary for receptor induction or coupling cannot be determined from the current results (Table 2); our experiments with inhibitors of RNA synthesis apparently suffer from the 'superinduction' ambiguities previously reported⁹.

Current models of receptor-adenylate cyclase interactions assume that functional coupling involves physical association of the components of the system and emphasise diffusion of the components in a fluid membrane⁴. Other investigators have suggested that microfilaments are involved in the modulation of certain receptors¹⁰ and that microfilaments may constitute structural bridges between microtubules and cell surface receptors¹¹. Our preliminary results with microtubule and microfilament disrupting agents (colchicid and cytochalasin B respectively) do not implicate these subcellular structures in the coupling of β -adrenergic receptors to adenylate cyclase (Table 2). It should be noted, however, that Ash and Singer¹² have shown the involvement of microfilaments in the mobility of concanavalin A receptors but could not demonstrate an effect of cytochalasin B on receptor mobility. Also Resch *et al.*¹³ have reported recently that microtubule-disruptive agents do not prevent initiation of lymphocyte activation by concanavalin A binding. Concanavalin A was inhibitory for functional β -receptor induction in HeLa, while stimulating the coupling of pre-induced receptors with adenylate cyclase (Table 2).

Concanavalin A binding to lymphocytes has been reported to immobilise many other receptors on the cell surface¹². In HeLa, concanavalin A binds extensively to the cell surface but does not inhibit ³H-alprenolol binding to β -adrenergic receptors (data not shown). HeLa cells remain viable for more than 24 h when glucose is omitted from the growth medium; in these conditions receptor induction is completely blocked and maintenance of pre-induced receptors is reduced by about half. Production of cyclic AMP in response to isoproterenol stimulation is greatly reduced by the omission of glucose from MEM (Table 2). These effects should be interpreted with caution since glucose-starved cells may be deficient in ATP and NADH (ref. 14). In coupling experiments, the addition of glucose to the cyclic AMP production assay mixture restored the cells' ability to produce cyclic AMP in response to the hormone (data not shown). Omission of Ca²⁺ from the medium was strongly inhibitory for receptor induction, while omission of serum had a less striking but significant effect. In contrast, the omission of either of these medium components had a stimulatory effect in coupling experiments.

These results clearly demonstrate that the β -adrenergic receptor can exist in a state in which it is functionally uncoupled from adenylate cyclase in HeLa cells. Exposure of cells to butyrate induces a striking increase in isoproterenol-stimulated adenylate cyclase activity. When high concentrations

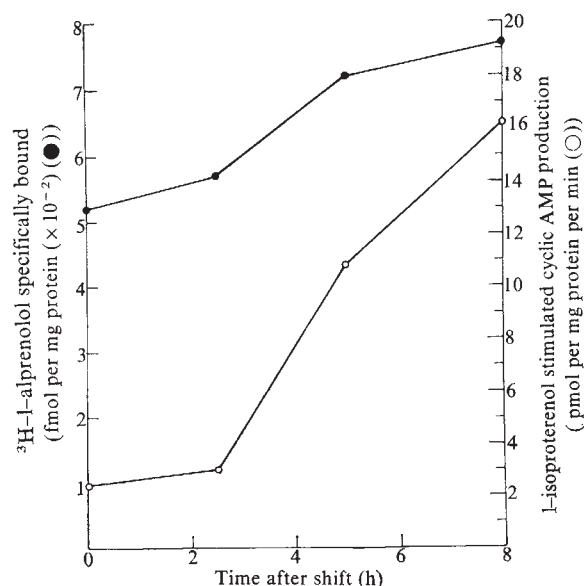


Fig. 2 Coupling of pre-induced β -adrenergic receptors with pre-existing adenylate cyclase. HeLa cells were cultured and collected and specific ³H-dihydroalprenolol and l-isoproterenol-stimulated cyclic AMP production determined as described in the legend to Fig. 1. Cells were incubated in medium containing 0.75 mM butyrate for 20 h and transferred to fresh medium containing 5 mM butyrate at zero time.

Table 2 Effects of inhibitors and/or omission of medium components on β -adrenergic receptor induction and coupling to adenylate cyclase

Addition/omission to medium + 5 mM butyrate	Induction of functional β -receptors		Coupling of β -receptors and adenylate cyclase	
	^3H -dihydroalprenolol bound (%)	Iso-stimulated cyclic AMP (%)	^3H -dihydroalprenolol bound (%)	Iso-stimulated cyclic AMP (%)
None	100	100	100	100
+ Cycloheximide (20 $\mu\text{g ml}^{-1}$)	14	< 2	12	41
+ Actinomycin D (1 $\mu\text{g ml}^{-1}$)	55	212	31	152
+ Cordycepin (10 $\mu\text{g ml}^{-1}$)	249	139	ND	ND
+ Colchicid (1 $\mu\text{g ml}^{-1}$)	91	91	101	98
+ Cytochalasin B (1 $\mu\text{g ml}^{-1}$)	84	91	124	126
+ Concanavalin A (25 $\mu\text{g ml}^{-1}$)	43	46	144	204
- Glucose	< 1	< 1	39	< 1
- Ca^{2+}	< 1	29	164	188
- Serum	56	53	281	127

For induction of functional β -receptors, HeLa cells were incubated for 24 h in medium, the medium replaced with medium containing 5 mM butyrate and the indicated addition or omission, incubation continued for 20 h and the cells collected. For coupling studies, cells were incubated 24 h in medium and the medium replaced with medium containing 0.75 mM butyrate; after 20 h incubation this was replaced with medium containing 5 mM butyrate, incubation continued for an additional 20 h and cells collected. ^3H -dihydroalprenolol binding and isoproterenol-stimulated cyclic AMP production were determined as before⁷. ND, Not determined.

(>2 mM) of butyrate are used, β -receptors are induced and become functionally coupled with pre-existing adenylate cyclase molecules within 10 h. At lower concentrations of the fatty acid, the receptors increase in number but do not become functionally coupled with the enzyme; a subsequent shift to the higher butyrate concentration leads to the onset of coupling after a brief lag. This system may prove useful for the study of receptor-adenylate cyclase interactions.

The effects of short-chain fatty acids on HeLa and other mammalian cells in culture are manifold. Among the phenomena elicited by the exposure of cells to butyrate are the following: (1) The inhibition of growth and alteration of morphology in HeLa¹⁵, Chinese hamster ovary¹⁶, and neuroblastoma cells¹⁷. (2) Increases in the ganglioside $\text{G}_{\text{M}3}$ and its biosynthetic enzyme in HeLa¹⁸⁻²⁰. (3) Increase in alkaline phosphatase activity in HeLa²¹. (4) Increased synthesis of the β -subunit of human chorionic gonadotropin in HeLa²². (5) Induction of erythroid differentiation in erythroleukaemic cells²³. (6) Induction of tyrosine hydroxylase and acetylcholinesterase activity in mouse neuroblastoma cells¹⁷. And (7) shape change correlated with elaboration of microfilaments and microtubules in transformed rat kidney cells²⁴.

In this report we have described at least two separate effects in HeLa cells: induction of β -receptor synthesis by low concentrations of butyrate and stimulation of receptor-adenylate cyclase coupling by higher concentrations.

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Thyroid hormone controls glucocorticoid action in cultured GH₁ cells

WE have demonstrated that L-triiodothyronine (T₃) stimulates growth hormone synthesis three to four-fold¹⁻³ and inhibits prolactin secretion¹ in cultured GH₁ cells, which constitute a rat pituitary cell line. Others have shown that cortisol also stimulates growth hormone production in GH₁ cells⁴. In GH₃ cells, a closely related cell line, cortisol stimulates growth hormone production four- to eightfold and inhibits prolactin secretion approximately fourfold⁵. Thus, thyroid and glucocorticoid hormones seem to control similar growth hormone and prolactin responses in GH₁ and GH₃ cells. Studies of the effects of glucocorticoids in these cell lines^{4,5}, however, have used commercial serum preparations containing physiological concentrations of thyroid hormone⁶. To examine the intrinsic response of these cells to glucocorticoid, therefore, it is necessary to use medium supplemented with serum which contains very low thyroid hormone levels. Using culture medium supplemented with hypothalamic calf serum, we have examined the influence of T₃ and cortisol on the induction of growth hormone synthesis in GH₁ cells. We report here that in the absence of T₃, the cells seem unresponsive to glucocorticoids, whereas in its presence cortisol markedly enhanced growth hormone production. The apparent requirement of T₃ for expression of the cortisol response seems independent of any influence of T₃ on total cell steroid receptor levels or the nuclear-cytoplasmic distribution of the steroid receptor, and of any effect of cortisol in modulating the level of the T₃ nuclear receptor.

Figure 1 illustrates a T₃ dose-response study for growth hormone production determined by radioimmunoassay with and without 100 nM cortisol after a 20-h incubation. At this time the cellular response to T₃ is maximal². T₃ alone stimulated a 7.5-fold increase, with a half-maximal response induced by 0.3 nM T₃. This is essentially the same as the value we obtained by quantifying directly the rate of synthesis using ³⁵S-methionine followed by immunoprecipitation of the intracellular labelled growth hormone³. At each concentration of T₃, 100 nM cortisol resulted in a twofold increase in growth hormone production with a half-maximal response also at 0.3 nM T₃. Cortisol therefore seems to potentiate the action of T₃ without altering the

T3 dose-response characteristics. In both cases, the biological dose-response relationships are in excellent agreement with the concentration of T3 (0.3 nM) that results in half-maximal occupancy of the thyroid hormone nuclear receptor in GH₁ cells³. This suggests that the T3-nuclear receptor complex controls a rate limiting event in the induction of growth hormone synthesis and that cortisol enhances the response initiated by T3.

Figure 2 illustrates a dose-response curve for growth hormone production as a function of cortisol concentration in the absence of T3 and in the presence of 0.3 nM and 5 nM T3, which respectively give half-maximal and full induction. Without added cortisol, the rate of growth hormone production induced by the two T3 concentrations is identical to that in the dose-response curve in Fig. 1. In the absence of T3, cortisol up to 5 μ M resulted in no increase in growth hormone production. With 5 nM T3, growth hormone production was maximal with 100 nM cortisol, and decreased slightly with higher steroid concentrations, probably due to a reduction in rates of synthesis of cell protein^{4,5}. At each T3 concentration, there was a half-maximal increase in growth hormone production at 18 nM cortisol, which agrees with the cortisol dose-response curve for

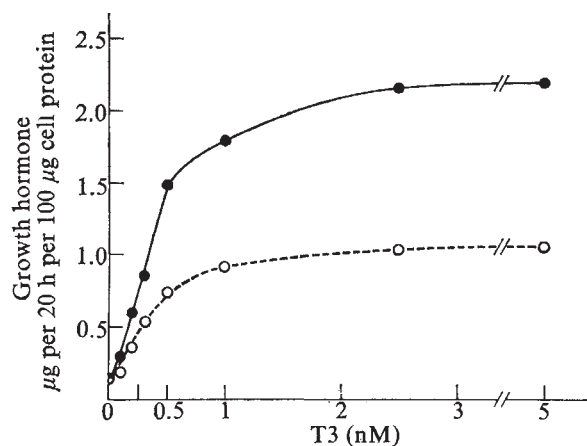


Fig. 1 Influence of cortisol on the induction of growth hormone production by T3. GH₁ cells were grown using Ham's F-10 medium supplemented with 2.5% foetal calf serum and 15% horse serum as previously described^{2,3,6}. The cells (150,000) were inoculated into wells of a 24-position Falcon multiwell plate, each with a surface area of 2 cm². After 48 h at 37 °C, the medium was exchanged with Ham's F-10 medium containing 10% hypothyroid calf serum and the cells were incubated for a further 48 h. The medium was then replaced with Ham's F-10 medium containing 10% hypothyroid calf serum and T3 was added at the concentrations indicated with (●) and without (○) 100 nM cortisol. The cell cultures were incubated for a further 20 h and growth hormone was quantified in the medium by radioimmunoassay¹. Because T3 (ref. 2) and glucocorticoids⁵ have no effect on the intracellular or extracellular degradation of growth hormone, and the intracellular pools are extremely low, the level of growth hormone determined in the medium by radioimmunoassay is an indirect measurement of rates of synthesis of growth hormone during incubation^{2,5}. After the medium was removed, each well was washed three times with 0.14 M NaCl at 4 °C and total cell protein was determined by the method of Lowry *et al.*²². Rates of production of growth hormone are expressed as µg of growth hormone produced per 20 h per 100 µg of cell protein. Each well contained approximately 60 µg of cell protein.

tyrosine aminotransferase reported for hepatoma tissue culture (HTC) cells⁷. The regulation of enzyme induction by glucocorticoids in HTC cells is mediated by steroid binding to a cytosol receptor⁸, followed by translocation of the steroid-receptor complex to the nucleus^{9,10}. Based on the dose-response studies of the growth hormone response, it seems likely that cortisol acts by a similar steroid receptor mechanism and in some way potentiates the action of T3 which functions as the primary inducer.

This is supported by studies using an anti-glucocorticoid in GH₁ cells. Samuels and Tomkins⁷ identified several compounds

(anti-inducers) which did not induce tyrosine aminotransferase in HTC cells but competitively inhibited the induction of the enzyme by cortisol. These findings were interpreted in terms of the interaction of these steroids with an allosteric receptor system concerned with induction of the enzyme. Two of these compounds, 17 α -methyltestosterone (17 α MeT) and progesterone, seem to act by inhibiting the binding of glucocorticoid to its receptor⁸, and evidence indicates that anti-inducers modify mRNA accumulation¹¹⁻¹³. Table 1 illustrates the effect of the anti-inducer 17 α MeT on the growth hormone response induced by T3 and by T3 plus cortisol. The growth hormone response

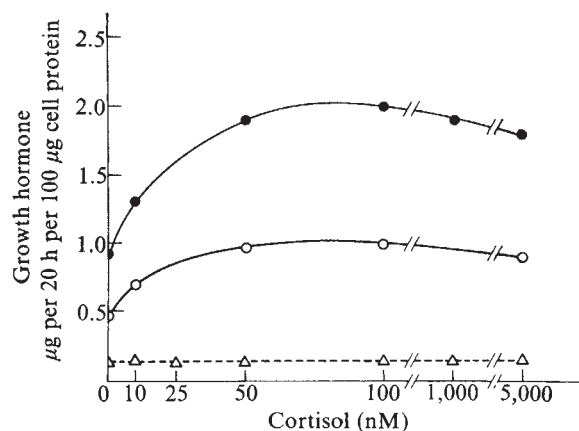


Fig. 2 Influence of T3 on cortisol induction of growth hormone production. The experiment was the same as in Fig. 1 except that the cells were incubated with cortisol without T3 (Δ), with 0.3 nM T3 ($\circ$) and with 5 nM T3 ($\bullet$).

was assessed in separate experiments using radioimmunoassay¹ to measure total hormone production, and by incubating cells with ³⁵S-methionine for 15 min followed by immunoprecipitation to quantify relative rates of synthesis^{2,3}. By either method, neither cortisol nor 17 α MeT alone or in combination, stimulated growth hormone synthesis. In each case, T3 stimulated an increase in the growth hormone response and this was stimulated threefold further by 100 nM cortisol. 17 α MeT decreased the growth hormone response in the cells incubated with T3 and cortisol to that of T3 alone, and only minimally influenced the response induced by T3 alone. Rates of synthesis of total cell protein were similar in all cases, with the exception of a slight reduction with cortisol alone or T3 plus cortisol, which is consistent with previous reports^{4,5}. These results support the concept that T3 is the primary inducer of growth hormone synthesis and cortisol potentiates the T3 response by a mechanism which involves the glucocorticoid receptor.

Growth hormone induction by T3 seems to be initiated by the association of T3 with a receptor localised in the cell nucleus^{2,3,14}, and seems to be explained by the accumulation of growth hormone mRNA². After 24 h, 5 nM T3 results in reduction of the thyroid hormone nuclear receptor to 40% of the initial value³. Therefore, the potentiation by cortisol of the response to T3 might be due to prevention of T3-mediated reduction of the thyroid hormone nuclear receptors resulting in an enhanced cellular response to T3. Stimulation of growth hormone synthesis by cortisol plus T3 might also be due to a T3-mediated increase of the glucocorticoid receptors.

Table 2 shows investigation of these possibilities. As before³, 5 nM T3 resulted in a 40% reduction in its homologous nuclear receptor population. Cortisol did not prevent receptor depletion by T3, and therefore the potentiation by cortisol of the response to T3 cannot be explained by interference in the reduction of the thyroid hormone nuclear receptors by T3.

T3 also had no effect on the level of the total glucocorticoid receptor or the nuclear-cytoplasmic distribution of the steroid

Table 1 Influence of T3, cortisol and 17 α MeT on synthesis of growth hormone

Additions	Growth hormone production (radioimmunoassay, μ g per 100 μ g cell protein per 20 h)	Growth hormone synthesis (c.p.m. per 100 μ g DNA $\times 10^{-2}$)	Total cell protein synthesis (c.p.m. per 100 μ g DNA $\times 10^{-4}$)
None	0.56	688	1,051
Cortisol, 100 nM	0.50	617	800
17 α MeT, 10 μ M	0.48	700	1,200
Cortisol, 100 nM plus 17 α MeT, 10 μ M	0.49	720	1,125
T3, 5 nM	2.60	1,502	1,100
T3, 5 nM plus 17 α MeT, 10 μ M	2.10	1,252	1,190
T3, 5 nM plus cortisol 100 nM	7.00	3,924	960
T3, 5 nM plus cortisol, 100 nM plus 17 α MeT, 10 μ M	2.50	1,304	1,132

The protocol was as described in the legend to Fig. 1. Additions were as indicated. The cells were incubated for 20 h and growth hormone production was determined by radioimmunoassay of the growth hormone content of the medium. Rates of synthesis of growth hormone were estimated in a separate experiment using the same protocol as in Fig. 1, except that 1×10^6 cells were initially inoculated into 25-cm² Falcon T flasks³. After incubation for 24 h at 37 °C with compounds indicated, the cells were incubated with ³⁵S-methionine (50 μ Ci ml⁻¹, 635 Ci mmol⁻¹) for 15 min. Total cell protein synthesis, and growth hormone synthesis quantified by specific immunoprecipitation of intracellular labelled growth hormone, was measured as before^{2,3}. Cell DNA content was determined by the method of Burton²³ and cell protein by the method of Lowry *et al.*²². Each point represents the mean of three determinations and each sample did not vary more than 5% from the mean.

receptor. The estimated level of glucocorticoid receptor binding sites per cell (approximately 12,000) agrees with the values reported for other cell systems¹⁵. The percentage of total glucocorticoid receptor identified in the nucleus, however, is approximately half of that reported for most other cell systems but is identical to that determined in glucocorticoid-sensitive lymphoma cells¹⁶. Therefore, the potentiation by cortisol of the response to T3 is not explained by one hormone modulating the receptor level of the other. It is possible, however, that T3 modifies a biologically relevant subset of nuclear association sites for the glucocorticoid receptor and that this is not identified by measuring the total nuclear associated glucocorticoid receptor.

Evidence indicates that glucocorticoids act at the pre-translational level to increase the rate of accumulation of

mRNA^{11-13,17,18}. When medium was used which was not devoid of thyroid hormone, glucocorticoids induced a concomitant increase in growth hormone production and growth hormone mRNA in GC cells, a cell line closely related to GH₁ cells^{19,20}. This suggests that the enhancement by cortisol of the response of GH₁ cells to T3 occurs at a level proximal to translation. Therefore, GH₁ cells provide a system for the examination of the initiation and potentiation of a specific response which seems to be regulated at the level of the cell nucleus. Because glucocorticoids induce tyrosine aminotransferase in HTC cells in serum-free conditions, thyroid hormone does not seem to be important for steroid action in HTC cells^{7,21}. Whether our observations with GH₁ cells of the interrelationship between thyroid hormone and glucocorticoid apply to other systems responsive to these hormones remains to be examined.

Table 2 Quantification of T3 and glucocorticoid receptors in GH₁ cells

Incubation	Nuclear T3 receptors (fmol per 100 μ g DNA)	T3 receptor binding sites per nucleus	Glucocorticoid receptors (fmol per 100 μ g DNA) Cytoplasm Nuclei		Glucocorticoid receptor binding sites per cell
Control	180	17,800	121	35.9	12,200
T3, 5 nM	110	10,877	121	34.9	12,100
T3, 5 nM plus cortisol, 100 nM	100	9,888	—	—	—

The protocol was as described in the legend to Fig. 1 except that 3×10^6 GH₁ cells were inoculated into 75-cm² Falcon T flasks. After incubation of cells in the presence of medium containing 10% hypothyroid calf serum for 48 h, either 5 nM ¹²⁵I-T3 or 5 nM ¹²⁵I-T3 plus 100 nM of non-radioactive cortisol was added for 24 h while a parallel set of flasks served as controls. One hour before the cells were collected, 5 nM ¹²⁵I-T3 was added to control flasks to determine the level of receptor present in cells not exposed to T3 during the 24 h (ref. 3). The T3 bound to nuclei was determined as before^{3,14}. The magnitude of nonspecific binding was determined by simultaneous incubation with a 500-fold molar excess of non-radioactive T3. This value represents less than 5% of the total T3 bound³ and was subtracted from the total to give the values above. DNA was quantified by the method of Burton²³. In other flasks, cells were incubated with 5 nM non-radioactive T3, while a parallel group of flasks served as controls. Glucocorticoid receptor levels were then determined by incubating GH₁ cells with 100 nM ³H-cortisol (91 Ci mmol⁻¹) with and without a 500-fold molar excess of non-radioactive cortisol for 1.25 h at 37 °C (ref. 24). The flasks were chilled by partially submerging them in ice for 15 min and the cell monolayers were rinsed three times with 10 ml of 4 °C 0.14 M NaCl. All further procedures were carried out between 0 and 4 °C. The cells were homogenised in buffer containing 0.25 M sucrose–20 mM Tris-HCl–1.1 mM MgCl₂ (pH 7.6 at 25 °C)¹⁴ and the samples were centrifuged at 800g for 10 min and nuclei were prepared from the resultant pellets with Triton X-100 as before^{3,14}. Purified nuclei were dissolved in 0.4 ml of 0.4 N NaOH and the ³H-cortisol bound was determined with a liquid scintillation counter using Aquasol solution. The labelled cortisol bound in the cytoplasmic fraction was determined by incubating triplicate 1-ml fractions with 2 mg of Norit A charcoal and 0.2 mg of Dextran (Sigma, molecular weight 500,000) for 2 min followed by centrifugation at 2,000g for 5 min (ref. 24). The derived supernatant fractions were analysed for labelled cortisol binding after the samples had been dissolved in Aquasol. ³H-cortisol binding to the nucleus or cytoplasmic fraction, bound to receptor, was calculated by subtracting the total labelled cortisol bound from that determined in the presence of a 500-fold molar excess of non-radioactive cortisol. These results represent the mean of a triplicate determination and each sample did not vary more than 8% from the mean. DNA was determined²³ on the nuclear fraction and the results are expressed as fmol bound per 100 μ g of DNA. The number of receptor binding sites per cell was based on the fact that 1×10^6 cells contain 13 μ g of DNA (ref. 14).

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Muscarinic antagonists block cone to horizontal cell transmission in turtle retina

As in other vertebrate retinas, the cone photoreceptor cells of the turtle are connected by complex synaptic endings with both horizontal and bipolar cells¹. The cone endings release their transmitter continuously in the dark, and light stimulation suppresses this release^{2–7}. The exact nature of the synaptic transmitter released by the cones is unknown but it is known to depolarise the horizontal cells^{5,8}; to depolarise the 'hyperpolarising' bipolar cells^{5,6} and to hyperpolarise the depolarising bipolar cells^{5,6}. It can also be foreseen that any interference with either the release or the postsynaptic action of this transmitter will cause an hyperpolarisation of both the horizontal and the hyperpolarising bipolar cells and a depolarisation of the depolarising bipolar cells, as well as the block of the light responses of the three cell types. Lam⁹ has shown that isolated cones of the turtle retina incubated in the presence of labelled precursors of acetylcholine (ACh) were able to synthesise ACh. In other species, however, assays of choline acetyltransferase activity in different layers of the retina showed a relatively low concentration of the enzyme in the outer plexiform layer (see ref. 10 for review) and ACh application to horizontal cells had no appreciable effects in carp and gekko retinas¹¹. Yazulla and Schmidt¹² recently reported that binding sites for ¹²⁵I-labelled α -bungarotoxin (a snake venom which specifically binds to ACh nicotinic receptors) can be demonstrated by autoradiography in the retina of goldfish and turtle and that the majority of these binding sites are located in the outer plexiform layer. In an attempt to re-evaluate the possibility that ACh is

the neurotransmitter liberated by cones, we studied the effects of some ACh agonists and antagonists on the cone to horizontal cell transmission. Our most interesting finding is that agents known to block the ACh muscarinic receptors block preferentially, and very probably at the post synaptic level, the synaptic transmission from the cone to the horizontal cell. Moreover, the same agents do not block the transmission from the cone to the hyperpolarising bipolar cells.

The eye was removed from the turtle, *Pseudemys scripta*, cut obliquely behind the iris and the vitreous fluid was drained. The eye cup was then mounted and fixed in a suitable chamber and perfused during the whole experiment with a bicarbonate-Ringer solution¹³ (4–5 ml per min, 110 mM NaCl, 2.6 mM KCl, 2 mM MgCl₂, 22 mM NaHCO₃, 2 mM CaCl₂, 10 mM D-glucose) continuously bubbled with a 95% O₂ + 5% mixture, so as to be kept at pH 7.8. The drugs used were dissolved in the Ringer solution and applied to the retina through the perfusing system through a multi-way tap. Intracellular recordings were performed using single high resistance micro-pipettes (300–600 M Ω) filled with 4 M potassium acetate. The retina was stimulated with a double beam photostimulator, the light stimuli were images of circular apertures 50–3,600 μ m in diameter. The light sources were tungsten lamps, and neutral density filters were used to reduce their intensity. The total flux density of unattenuated light at the retinal level was 6×10^8 μ W cm⁻² with wavelengths of 400–800 nm.

Retinas are very rich in acetylcholinesterase, particularly in the inner layers¹⁰, so we thought it very unlikely that ACh could reach the outer plexiform layer without being hydrolysed. We therefore applied carbachol (an ACh analogue not hydrolysed by the esterase) to the retina and studied its effects on the L-horizontal cells. The membrane potential of L-horizontal cells varies between two extreme levels, a dark potential level and a light potential level; carbachol at high concentrations (1–5 mM) causes a depolarising shift of both the dark and light membrane potential levels, thus markedly decreasing the light responses of L-horizontal cells. This effect was reversible and disappeared after washing, but this action of carbachol was not observed in all experiments.

In spite of the irregularity of the action of this agonist, we pursued our pharmacological study by applying to the L-horizontal cells a series of ACh antagonists. The nicotinic antagonists, gallamine (flaxedil), hexamethonium and tetraethylammonium in concentrations up to 5 mM and tubocurarine at up to 1 mM neither altered the dark potential nor reduced the light responses of the horizontal cells. The muscarinic antagonists, in contrast to the nicotinic antagonists, showed a consistent blocking action. As seen in Fig. 1, application of atropine (1–5 mM) caused a progressive hyperpolarisation and, in parallel, a marked and progressive reduction of the light responses (Fig. 1c). Atropine also slowed down the hyperpolarising phase of the responses and caused the appearance of a small off-depolarisation (Fig. 1d). The responses became almost completely blocked after 15–20 min of atropine perfusion (Fig. 1c, d). The action of atropine could be reversed, but only by prolonged washing. Effects similar to those produced by atropine were produced by other muscarinic antagonists such as hyoscine and propanthine, but their blocking efficacy was lower at similar dose ranges.

The effect of other ACh agonists on L-horizontal cells was also studied. The nicotinic agonist, decamethonium (5 mM), was inactive. The muscarinic agonist, pilocarpine (1 mM), depolarised the L-horizontal cell membrane potential and reduced the amplitude of the light responses, and at higher doses, produced a mixture of antagonistic and agonistic effects. Amongst the other muscarinic agonists tested, arecoline (1–5 mM) behaved as an antagonist, blocking the responses as atropine does, and acetyl- β -methylcholine was completely ineffective.

We also explored the action of anticholinesterase agents on the L-horizontal cells. Eserine (2–5 mM) caused a depolarising shift of the dark potential which was followed by a

hyperpolarisation. This latter phase was accompanied by a marked decrease of the light response, the development of a large off-depolarisation and a marked depolarising shift of the membrane potential level in the light. Analogous but milder effects were produced by similar concentrations of neostigmine and edrophonium.

The data relating to the action of atropine and other muscarinic antagonists deserve a more detailed analysis. Thus atropine was found to show some presynaptic action, since it caused both a slight hyperpolarisation of the cones

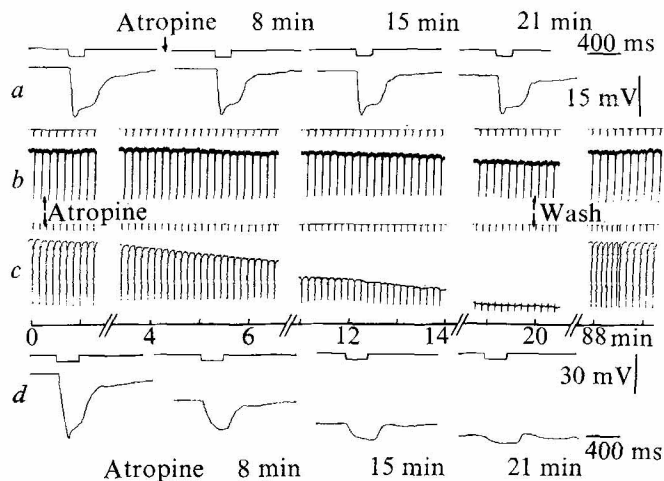


Fig. 1 Intracellular recordings from a cone (*a, b*) and from an L-horizontal cell (*c, d*) of turtle retina. The trace above each recording gives the timing of the light stimulations. Both cells were stimulated with white light (relative intensity, -1.3), the cone with a $155 \mu\text{m}$ diameter light spot whereas the spot used for the horizontal cell had a diameter of $3,600 \mu\text{m}$. The low speed recordings in (*b*) and (*c*) have a common time base as shown below the (*c*) trace. Application of atropine 5 mM (arrows) caused in the cone (*b*) a slight hyperpolarisation and reduction of amplitude of the light response, which became diminished by one-third. In (*c*), atropine produced a hyperpolarisation of the L-horizontal cell and almost blocked the light response. After recordings (*b*) and (*c*) prolonged washing resulted in a recovery of the response of both cells. *a* and *d* correspond respectively to high speed recordings from a cone and L-horizontal cell. The light responses of both cells were recorded (from left to right) just before, and after 8, 15 and 21 min of atropine application. Note that in (*d*) the almost complete block of the horizontal cell light response and the development of an off-depolarisation.

(Fig. 1*a, b*) and a reduction of their response to light. But, the effect of atropine on the L-horizontal cells cannot be explained by this presynaptic effect alone: Fig. 1 shows that atropine reduced the cone response by at most one-third of its original amplitude, whereas the L-horizontal cell response was almost completely suppressed.

Moreover, and more importantly, atropine was found to have little effect both on the dark potential and on the response of the hyperpolarising bipolar cells to light spots which stimulated the centre of their receptor field (that is, which activated the direct connection between the cone and the bipolar cell). This observation was confirmed in 10 different hyperpolarising bipolar cells (recognised as such by the criteria set by Schwartz¹⁴ and Richter and Simon¹⁵). Figure 2*a* corresponds to a typical response of one of these bipolar cells in response to a central light spot, showing both the membrane noise decrease during the hyperpolarisation and an off-depolarisation. The response of Fig. 2*b* was recorded from the same cell after more than 25 min of atropine application, and it is evident that the response was only slightly reduced, and that the membrane noise was not altered, the small reduction of the light response amplitude being probably a consequence of the aforementioned diminution of the cone response (moreover, in two cases we observed

an actual increase of the bipolar response under atropine). We conclude from this experiment that atropine does not affect the connection between the cones and the hyperpolarising bipolar cells, and therefore the effect of the drug on the L-horizontal cells cannot be presynaptic. If the transmitter release from the cones were actually decreased, the bipolar cells would have been hyperpolarised, and both a decrease of the membrane noise in the dark and a block of their hyperpolarising responses would have been observed.

From the results presented here we conclude that atropine and other muscarinic agents block the cone to L-type horizontal cell synapses by acting mainly at the postsynaptic level. It is difficult, at this stage, to relate these results to a possible transmitter role of ACh in the cone synapses. The effective concentrations of the agonists and antagonists used were very high, and we do not know if the need for such high concentrations is due to the existence of diffusion barriers or because we are dealing with nonspecific effects of muscarinic drugs which only appear at high doses. Diffusion barrier mechanisms would also account for the irregularity of effect of some of the choline derivatives as seen by us and by other workers.

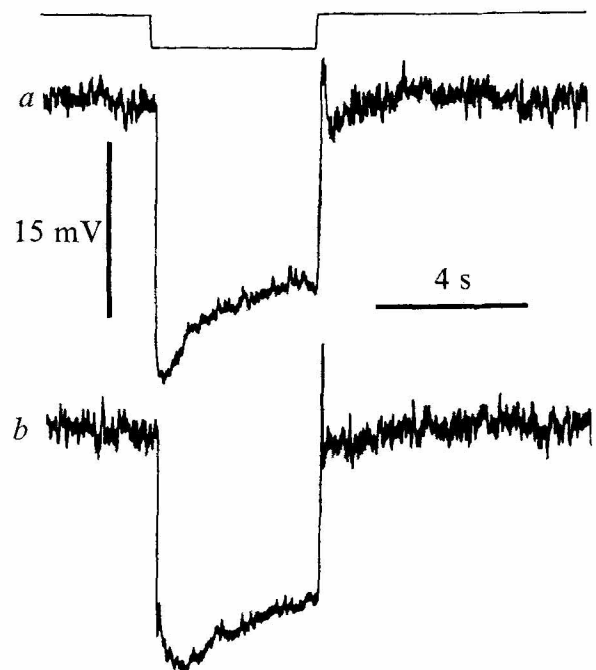


Fig. 2 *a*, Control hyperpolarising response of a hyperpolarising bipolar cell to the stimulation of the centre of its receptor field with a white light spot (diameter $600 \mu\text{m}$, relative intensity -2.3). Note the reduction of the membrane noise during the hyperpolarisation. *b*, Response from the same cell to an analogous stimulation, recorded after applying atropine 5 mM to the preparation for 25 min. The response was slightly decreased in amplitude but no alteration in membrane noise was observed.

Finally, it is interesting to note that if ACh were the transmitter released from the cones our results imply that it should act on different receptors on the L-horizontal cells and on the hyperpolarising bipolar cells.

The main finding which we want to emphasise is that atropine blocks preferentially the synaptic responses of L-horizontal cells at the postsynaptic level. In the following paper¹⁶ this property of atropine is used to analyse some of the known lateral cell interactions in the outer plexiform layer of the turtle retina.

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Lateral interactions in the outer plexiform layer of turtle retinas after atropine block of horizontal cells

IN the retina of the turtle and of other vertebrates, L-type horizontal cells are thought to play an interneuronal role ensuring long-distance linking between other retinal cells which synapse in the outer plexiform layer. L-type horizontal cells have been thus implicated in: (1) the feedback loop through which peripheral stimulation evokes a depolarising synaptic potential in cones^{1–3}, (2) the cell network which participates in the generation of responses of opposite polarity in chromatic C-type horizontal cells^{2,4,5} and (3) in the centre surround organisation of bipolar cells, where horizontal cells have been postulated to mediate the responses to peripheral stimulation^{6–8}. We have shown previously⁹ that atropine and other muscarinic antagonists block the cone to L-horizontal cell transmission, but do not block the cone to hyperpolarising bipolar cell transmission. Taking advantage of this pharmacological distinction, we investigated the participation of the L-horizontal cells in the above mentioned cases of lateral interaction at the outer plexiform layer. We found that atropine blocks both the feedback depolarising potential of the cones and the responses of the C-type horizontal cells, having eliminated previously to this block the opposition between the responses to different colours. At variance with these blocking actions, atropine does not block, and even increases, the depolarising surround responses of the hyperpolarising bipolar cells. These findings confirm the conclusions of previous authors that L-type horizontal cells are involved in generating both the feedback depolarising potential in the cone and the opposite responses of C-type horizontal cells to different colours. Contrary to current ideas, they demonstrate that the L-horizontal cells are not responsible for the depolarising responses of the hyperpolarising bipolar cells to the stimulation of the periphery of their receptor field.

The preparation and the general experimental procedures were similar to those described previously⁹. Atropine was used in these experiments at 5 mM. The retina was stimulated with concentric circular and annular stimuli of white or monochromatic light using apertures varying between 50 and 3600 μm in external diameter, presented either alternately or simultaneously.

Figure 1 corresponds to one of a series of experiments on the cone feedback mechanism. The recordings of Fig. 1 were obtained from a cone responding to central spot

illumination with a typical 15-mV amplitude hyperpolarisation (Fig. 1a) and which responded to a very bright annular flash with a fast hyperpolarisation (Fig. 1b). The stimulation of this cone by a combination of both spot and annulus in appropriate timing (Fig. 1c), resulted in the appearance of a depolarising feedback potential with the characteristics described by Baylor *et al.*¹. After bathing the retina in atropine for 15–20 min, the feedback depolarising potential of the cones disappeared completely and the annular stimuli then only evoked a small hyperpolarisation (Fig. 1f). It is well known that the feedback depolarisation is rather labile and that, in many cases, it may disappear spontaneously. At least in four cones studied (among them, the one recorded in Fig. 1) it was possible to observe a complete recovery of the feedback potential after removal of the atropine. These experiments on the feedback depolarising potentials confirm the existing evidence² on a L-horizontal cell participation in these responses.

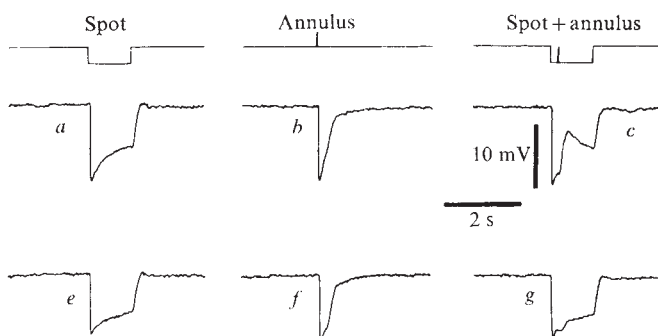


Fig. 1 Atropine effect on the feedback depolarising potential of a turtle cone. The top row of traces shows the timing and duration of the light stimuli. *a–c*, Control recordings in a cone. In (*a*) the cone responds with an hyperpolarisation to a centred spot of white light (diameter 250 μm , relative intensity -2.3). In (*b*) the same cone was stimulated with a bright annulus (internal diameter 430 μm , external diameter 3,600 μm ; relative intensity -0.3) which caused a fast hyperpolarisation (which probably resulted from light scattering to the centre). The recording in (*c*) shows the appearance of a feedback depolarising potential when the annulus is presented during the centre stimulation, 180 ms after the onset of the latter. The recordings (*d–f*) were obtained from the same cone, using the same stimulation parameters, after perfusing the preparation with atropine 5 mM. Note that in (*f*) the depolarising feedback potential has been blocked and that flashing of the annulus in the same temporal relation with the centre stimulation only evokes a small hyperpolarisation due to light scattering.

Another lateral interaction mechanism in which the atropine blocking experiments support the idea of a participation of L-horizontal cells, concerns the organisation of the colour responses of the C-type horizontal cells. Figure 2 shows recordings from a green-red coded C-type horizontal cell which was alternately stimulated with large spots of red and white light. White flashes resulted in an hyperpolarisation, while red (650 nm) flashes depolarised the cell (Fig. 2a). Application of atropine to the preparation caused a slowly developing hyperpolarisation of the C-type horizontal cell and a decrease in the amplitude of both kinds of responses, although in the first minutes the hyperpolarising responses decreased faster than depolarising ones. After 8 min of atropine application (Fig. 2b) both responses were equally diminished but it could be seen that the responses to red flashes became biphasic, the depolarisation appearing then preceded by a short hyperpolarisation. Progressive blocking of the C-horizontal cells was accompanied by a reversal of the responses to red stimuli, and Fig. 2c shows that after 15 min of atropine perfusion both types of stimuli evoked very small hyperpolarisations. The atropine-induced reversal of the responses to red flashes can be interpreted by assuming that in normal conditions the depolarising red response masks a direct

hyperpolarising response due to stimulation of the green cones, which reappears when L-horizontal cells are blocked. This finding is consistent with the interpretation⁷ that the opposite responses of the red-green coded C-type horizontal cells involve L-horizontal cells interconnecting red and green cones.

The last problem of lateral interaction which we analysed with the aid of atropine is probably the most interesting one and concerns the organisation of the receptor field of the hyperpolarising bipolar cells. Figure 3 shows at left, a series of intracellular recordings from a hyperpolarising bipolar cell identified (as the group of ten we studied) by^{8,10}: (1) the high sensitivity to dim light, (2) the centre-surround organisation, (3) the typical off-depolarisation after prolonged stimuli and (4), the attenuation of the membrane noise during light centre response¹¹. In the experiment of Fig. 3, the hyperpolarising bipolar cell responded by a 20 mV hyperpolarisation to a centrally located spot stimulus and by a few mV depolarisation to a concentric annular flash (Fig. 3a). The recordings on the right side of Fig. 3 (d, e) were obtained from L-horizontal cells in the same preparation. The right top recording (Fig. 3d) is from an horizontal cell recorded before finding the bipolar cell at left and which was stimulated with a large spot with the same illumination intensity parameters used for centre stimulation of the bipolar cell. The recording of Fig. 3e is from one of a group of L-horizontal cells penetrated immediately after removing the pipette from the bipolar cell (that is, after an exposure of the preparation to atropine for more than 20 min). As described in the previous paper, the hyperpolarising responses of the hyperpolarising bipolar cells to a central light spot were only slightly reduced after atropine application (Fig. 3, middle and bottom left recordings), and developed complex off-phases. Moreover, the depolarising responses to peripheral stimuli were not blocked, but even increased after 20 min of atropine application (Fig. 3c). This observation was repeated on 10 hyperpolarising bipolar

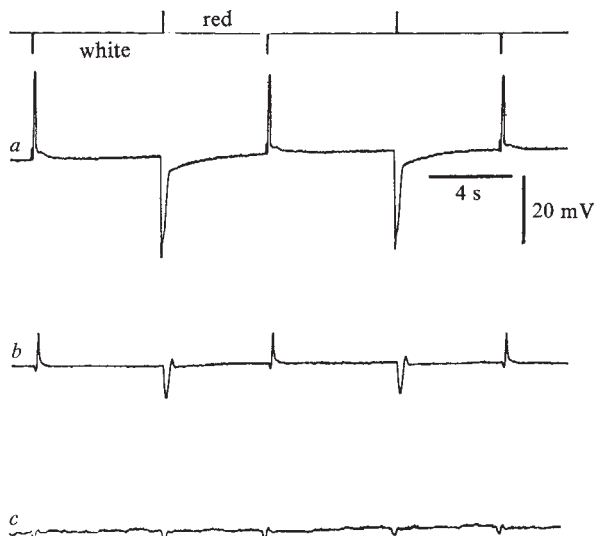


Fig. 2 Atropine effect on the responses of a green-red coded C-type horizontal cells. The top tracing marks the timing of the light stimuli, the red stimuli downwards, the white ones upwards). In (a) (control), the C-horizontal cell responded with depolarisations to stimulations with large spots of red light (650 nm, delivering 2.5×10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$) and with hyperpolarisations when similar size spots of white light (relative intensity -1) were used. b, After applying atropine for 9 min, the C-horizontal all became hyperpolarised and both types of response seemed diminished in amplitude, but note that the hyperpolarising responses to red stimuli became preceded by a small short hyperpolarisation. c, After 15 min of atropine application, the responses were markedly reduced in amplitude and the red stimuli evoked only small hyperpolarising responses.

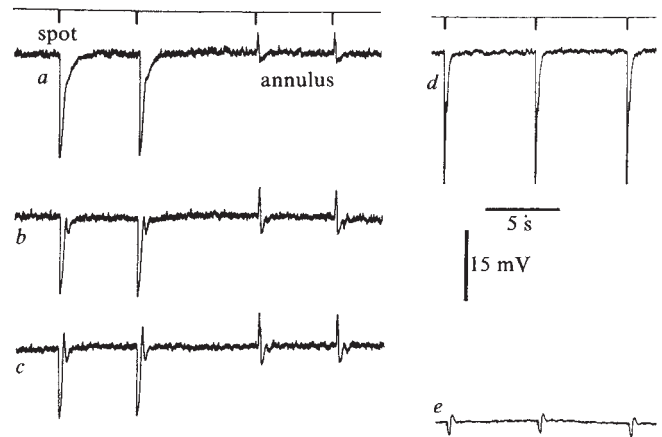


Fig. 3 At left (a, b, c), recordings from a hyperpolarising bipolar cell and at right (d, e) recordings from two different horizontal cells. The top traces on both sides mark the timing of the light stimulations. At left the two first stimuli in each row were centred light spots (diameter 380 μm , relative intensity -3) and the two following stimuli were white light annuli (internal diameter 500 μm , external diameter 3,600 μm ; relative intensity -5). a, Control recordings of the bipolar cell response to the spot and annulus. b, After 10 min of atropine application, the central responses were slightly diminished and showed off-hyperpolarisations, whereas the responses to the periphery were increased in amplitude. c, After 20 min of atropine application, the centre responses were not changed, except for the increasing complexity of the off responses, while the amplitude of the responses to the annulus had further increased. d, Recording from an L-horizontal cell impaled just before penetrating the bipolar cell at left, showing a hyperpolarising response to white light large spots (diameter 3,600 μm , relative intensity -3). The recording in (e) is from another L-horizontal cell penetrated at the end of the experiment, just after removing the pipette from the bipolar cell at left, while the preparation was still bathed in atropine. Notice the block of L-horizontal cell responses after more than 25 min atropine application.

cells and in two of them the atropine treatment was maintained for more than 30 min. Since at the end of such prolonged applications of atropine, the responses of the L-horizontal cells of the same preparations were almost completely blocked (as it is the case in Fig. 3e), a participation of L-horizontal cells in the generation of the depolarising responses of hyperpolarising bipolar cells to the stimulation of the periphery of their receptor field can be excluded, except probably for some kind of tonic inhibition which would account for the increase of the surround responses under atropine.

It seems difficult at present to suggest what cellular elements of the retina are responsible for the antagonistic surround responses in the hyperpolarising bipolars of turtle retina. The two main possibilities, if this interaction takes place in the outer plexiform layer, are that either the periphery cones (through the long processes described by Lasansky¹² in *Pseudemys retina*) or other bipolar cells are responsible for generation of the surround responses of hyperpolarising bipolar cells.

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Membrane control of cardiac contractility

ALL myocardial cells contract in every cardiac cycle and the contractility of the heart cannot be modulated as in skeletal muscle by varying the number of active cells, so each cardiac cell must be capable of a broad range of contractile states. Such versatility could result from changes in the action potential, in the amount of Ca^{2+} released for activation of the contraction, or in the contractile proteins themselves. Phosphorylation of the inhibitory subunit of troponin seems to occur in parallel with the increase in contractility of isolated hearts perfused with catecholamines^{1,2}, and studies using isolated proteins indicate that the phosphorylation may be associated with a change in ATPase activity^{3,4}, but a direct demonstration that the functional properties of contractile proteins themselves can be altered in a manner compatible with a physiologically important regulatory mechanism has not yet been produced. We report here studies of cardiac cells with membranes that have been made hyper-permeable, in which the response of the contractile system to Ca ions, the presumed trigger for activation of contraction, may be altered markedly with respect to both the concentration of calcium required for activation and the maximum generated force by the microenvironment of the contractile proteins and by reactions involving the cell's membranes. Cyclic nucleotides may be involved in some of these regulatory reactions.

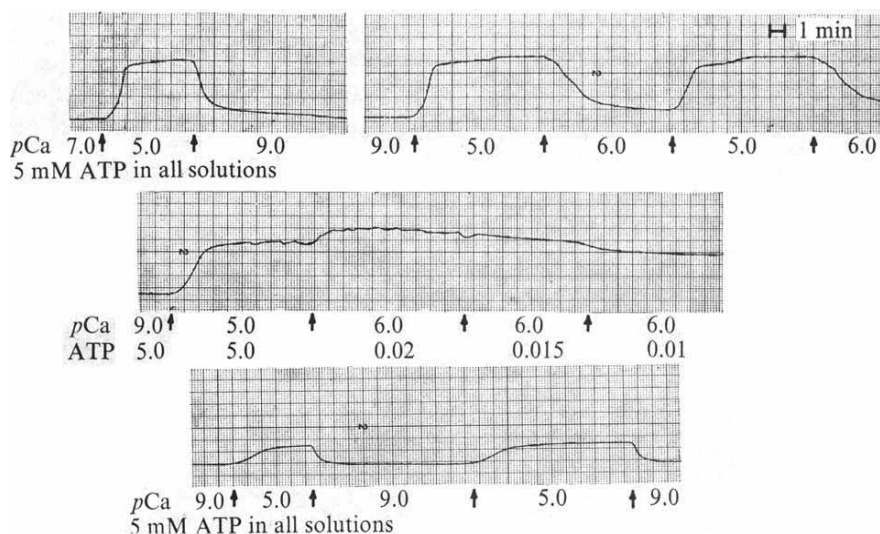
For these experiments a bundle of cardiac cells from a rat right ventricle which had been 'functionally skinned' by chelating membrane calcium with EGTA was used⁵. The sarcolemma of these cells, though highly permeable to small molecules and ions, still retains large molecules such as creatine phosphokinase and pyruvate kinase which can easily be lost when the sarcolemma is removed. Proteins like adenylate cyclase and protein

kinase, which may be involved in the regulation of the contractile system and which are relatively tightly associated with structural elements of the cell⁶, should therefore be present in the skinned fibre and presumably responsive to changes in concentration of small inhibitory or stimulatory cofactors such as Ca^{2+} , Mg^{2+} and ATP in the bathing medium.

The force produced at any concentration of Ca^{2+} from 10^{-9} M, a level considerably below the normal threshold for activation, to 10^{-5} M, the optimal concentration for activation, can be increased by lowering the concentration of MgATP, and the maximum Ca -activated force in 0.02 mM MgATP is about twice that produced in 5 mM ATP, the approximate intracellular concentration. In addition to an increase in force production, which may be due to either more cross bridges bound to actin at a time or more force from each cross bridge, the threshold concentration of Ca required for activation is reduced, suggesting that the affinity of troponin for Ca has been enhanced. These observations are very similar to those made by Bremel *et al.*⁷, on isolated myofibrils from skeletal muscles, but comparable experiments with a mechanically skinned cardiac cell, in which the sarcolemma has been removed, have not been done at the very low concentrations of MgATP (ref. 8).

Exposure of the EGTA-skinned fibres for several minutes to 5–20 μM Ca in the presence of a very low concentration of ATP produces a marked change in the contractile properties of the fibres (Fig. 1). Force, which is normally well maintained in 5 mM ATP, falls, and this decline continues for several minutes after the ATP concentration has been returned to 5 mM, indicating that the combination of low ATP and high Ca acts through an intermediate reaction and not directly on the contractile proteins. After several minutes in 5 mM ATP the fibres stabilise, but their response to activating concentrations of Ca in 5 mM ATP is now much weaker. The extent of the inhibition, which can be almost total, depends primarily on the duration of the soak in the solution with low ATP and high Ca . The requirements for the inhibition of force generation are 1 μM Ca or more and 20 μM ATP or less, but some ATP must be present. If the membranes of the skinned fibres have been disrupted with the non-ionic detergent Brij for 45 min, a reduction of ATP to only 1–2 mM is necessary, but the Ca requirements remain unaltered. Apparently two reactions, one ATP

Fig. 1 Force record produced by a rat ventricular trabecula which had been functionally skinned by an overnight soak at 4 °C in a solution containing 10 mM EGTA, 4 mM MgAc_2 , 5 mM ATP and 120 mM K propionate. The numbers under the records indicate the $p\text{Ca}$ ($-\log[\text{Ca}^{2+}]$) and the concentration of ATP in the bathing solution. The force developed in 5 mM ATP during maximal activation is very reproducible in each of the first four exposures, but when the ATP concentration is lowered to 0.01–0.02 mM the force declines. After return to 5 mM ATP the force in optimal $p\text{Ca}$ is again stable, but it has been markedly reduced. The shape of the tension- Ca relation is the same after the exposure to low ATP as it was before; a uniform relative decrease in force at each concentration of Ca has occurred. In all solutions the concentration of free Mg^{2+} ions was held constant at 2 mM after taking into consideration its interactions with ATP, EGTA and creatine phosphate. The MgATP concentration was controlled by varying ATP concentration; in view of the large excess of Mg ions it was assumed that all ATP was in the form of MgATP. Ca^{2+} was buffered with 3 mM EGTA and ATP with 15 mM creatine phosphate. Creatine phosphokinase, although present in high activity in the tissue, was also added with the creatine phosphate. In most experiments ionic strength was maintained by varying the concentration of KCl, but the inhibition of contractility produced by the combination of low ATP and high Ca was not particularly sensitive to ionic strength. The phenomenon occurred in solutions with ionic strengths of approximately 0.7 to 1.5 times the usual value, which was approximately equivalent to 170 mM KCl. The pH was buffered at 7.0 with either 25 mM imidazole or Tris at the equivalent ionic strength, and the results were independent of the specific buffer which was used. The standard test solutions with different $p\text{Ca}$ that were used before and after the exposure to low ATP were identical in composition.



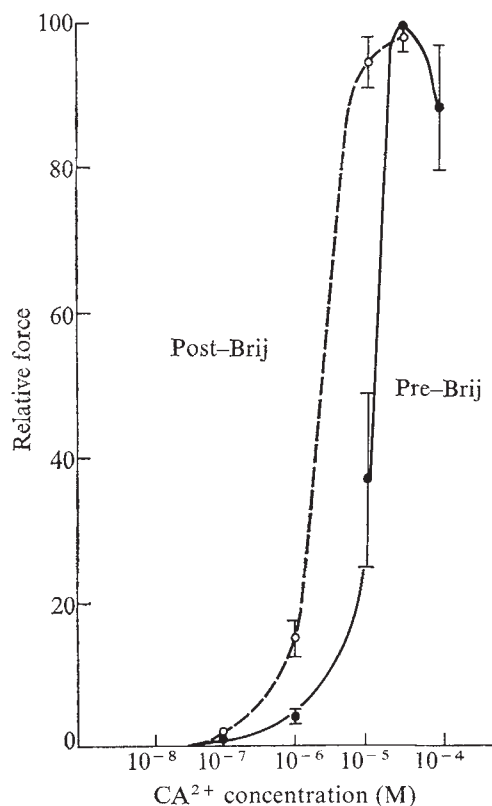


Fig. 2 The effect of the treatment of an EGTA-skinned rat ventricular bundle with Brij on the relation between force and Ca concentration. The solid curve indicates the values obtained in five preparations before Brij treatment and the interrupted curve, the values for the same muscles after 45 min in 0.5% Brij 58 followed by 60 min of washout in the standard bathing solution with no added Ca. The shift which is produced by Brij treatment has not been seen in skinned preparations where the surface membrane had been manually removed before the exposure to the detergent⁹. The Ca-tension relation found in the EGTA-skinned fibre after Brij treatment closely resembles what has been observed with the manually skinned cardiac muscle fibre⁹.

dependent and requiring membranes and the other Ca dependent and not needing membranes, are involved in the change in contractility.

Exposure to Brij 58 or another detergent, Triton X-100, increases the sensitivity of the contractile system to Ca by a factor of almost 10 without altering the basic shape of the relation between Ca and force (Fig. 2). This is in contrast to the absence of any effect of Brij on the tension-Ca relation in cardiac cells from which the surface membrane had been completely removed manually⁹. A similar shift of the curve along the Ca concentration axis is produced by 5 mM theophylline or an experimental phosphodiesterase inhibitor (Squibb 20009). Apparently a nucleotide cyclase located in the membrane is involved in the regulation of the Ca sensitivity of the contractile proteins.

The effect of low ATP and high Ca on skinned fibres can be modified by the addition of sarcolemma vesicles¹⁰ to the bathing medium. Their presence in the low ATP-high Ca solution prevents the decline in contractility, and their addition to a normal Ca-activating solution restores the original contractility to an inhibited muscle bundle for as long as the membrane vesicles remain in the bathing solution (Fig. 3). Addition of the membrane fraction to the solution bathing a non-inhibited skinned fibre causes little or no change in its contractile response to Ca. Since the preparation retains a functional diffusion barrier at its surface, the membrane vesicles are unlikely to be entering the cells and are more likely to be producing a substance which diffuses into the cytoplasm.

The capacity of the contractile system for generating force may, therefore, be controlled, at least in part, by the net result of two reactions. One reaction involving the cell membranes maintains a high capability for force generation, and the other, probably located in the myofibril, depresses the ability of the system to produce tension. The membrane reaction does not seem to involve the contractile system directly, but rather to suppress either the synthesis of an inhibitory molecule or the interaction of an inhibitory molecule with the contractile proteins. In view of the importance of the sarcolemma and the apparent inhibition by ATP concentrations below 20 μ M, the first reaction may be associated with the production of cyclic AMP and the requirement of the second reaction for ATP

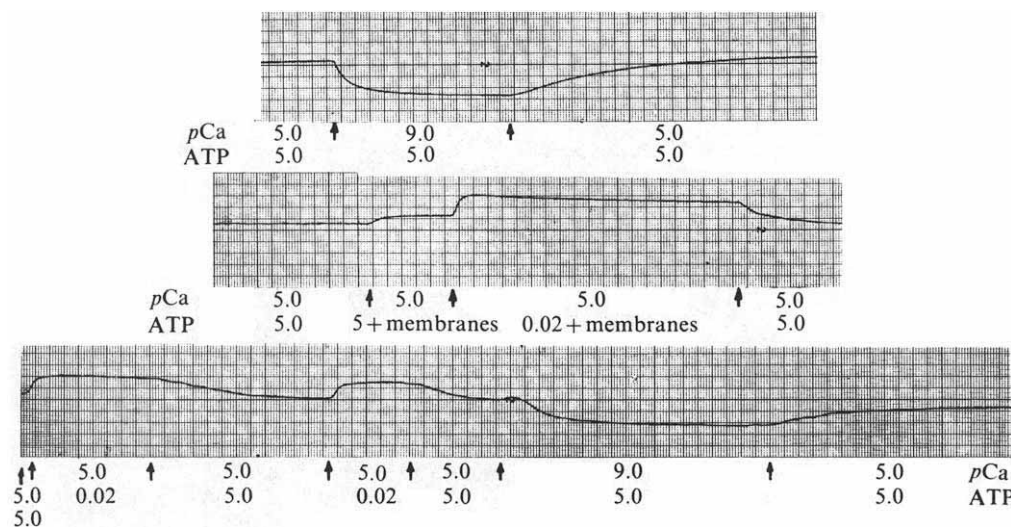


Fig. 3 The presence of isolated sarcolemmal vesicles in the bathing medium protects the EGTA-skinned rat ventricular cells from the negative inotropic effect of an exposure to the combination of low ATP and high Ca. Numbers under the force record indicate pCa and mM ATP. Force in optimal Ca and 5 mM ATP is reproducible. The exposure of the tissue to pCa of 5.0 and 20 μ M ATP does not inhibit the subsequent ability of the tissue to generate force on pCa of 5.0 when sarcolemma vesicles (prepared according to Lefkowitz and Haber¹⁰) are present in the bathing medium. Since the membranes which were added did not show any Ca-activated ATPase, major contamination with sarcoplasmic reticulum is unlikely. ATP concentration was buffered with 15 mM creatine phosphate, which was at least 5 mM in excess of what

was necessary to prevent any detectable sign of ATP limitation. Direct measurements of the bathing solution showed that the presence of the membranes caused a decline in creatine phosphate of only 1.5 mM every 30 min. The presence of 15 mM creatine phosphate at the beginning of a force measurement satisfactorily maintained the ATP concentration. The membrane fraction in the bathing solution represented an approximately 100-fold dilution of the concentration of membranes in the original ventricle, and consequently, its presence would contribute very little to the ionic strength or osmolarity of the bathing solution. The temporary protection from the presence of the membranes disappears with their removal as shown in the record, but in the absence of an exposure to low ATP without membranes, tension is well maintained for several hours after the exposure to low ATP with membranes. The small rise in force when membranes were added to the bathing solution was sometimes seen. It was completely reversible and was probably due to a temporary reversal of a small amount of existing inhibition of contractility.

suggests a Ca-regulated phosphorylation¹¹. Experimentally, however, the addition of cyclic AMP or cyclic GMP alone has failed to reproduce the effect of the low ATP-high Ca solution, the membrane fraction or Brij treatment. If our inferences about their involvement are valid, the cyclic nucleotides can be active only in very specific conditions, and alteration of contractile proteins is controlled by a pattern of molecules and not by a single molecular species. The sensitivity of the potentially responsive fraction of the contractile proteins to Ca is also regulated by the sarcolemma, possibly through a cyclic nucleotide controlled phosphorylation of the contractile proteins⁴.

The sensitivity of the contractile system to the concentration of ATP may represent a protective mechanism available to the cell for conserving energy for life sustaining reactions when the supply of oxygen or substrate is deficient. It would constitute a mechanism whereby the work of the tissue could be adjusted to its energy supply.

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Postsynaptic effects of a multi-action giant interneurone on identified snail neurones

A FEW examples of identifiable neurones which have widespread monosynaptic and multi-action effects on large numbers of postsynaptic cells have been found in gastropod molluscs^{1–3}. Such neurones are of considerable interest to neurophysiologists attempting to understand how the electrical activity of large networks of nerve cells can be integrated. We report here that the right pedal giant neurone (RPGN) of the pond snail, *Lymnaea stagnalis* (L.), is an interneurone with monosynaptic connections with many previously identified giant cells⁴ and cell clusters⁵ situated on the visceral and right parietal ganglia of *Lymnaea*. The RPGN mediates excitation on some cells, inhibition on others and biphasic effects (excitation followed by inhibition) on yet other cells.

Isolated brains from 1–9-g specimens of *L. stagnalis* were prepared for electrophysiological recording as previously described⁵, except that the commissure between the cerebral ganglia was cut and the ganglia spread apart. This procedure facilitated simultaneous electrophysiological recording from the RPGN in the right pedal ganglion and from its follower cells on the dorsal surfaces of the visceral and right parietal ganglia. Intracellular recordings were obtained from neuronal somata using conventional recording methods and 10–20-M Ω K₂SO₄ filled microelectrodes. Preparation of electrodes was greatly facilitated by use of triangular cross-section Pyrex electrode glass which allowed rapid, reliable filling of microelectrodes with electrolyte without previous addition of glass fibres.

The RPGN lies on the postero-ventral surface of the right pedal ganglion, medial to the statocyst and has a major soma diameter within the range 120–240 μ m in different snails. The positions of the cell bodies of neurones innervated by the RPGN are shown in Fig. 1. RPGN action potentials evoked by intracellular injection of depolarising current pulses via the recording electrode result in excitatory postsynaptic potentials (e.p.s.p.s)

in A group (Fig. 2a), the H cells (Fig. 2e) and K cells (Fig. 2b, c, d), inhibitory postsynaptic potentials (i.p.s.p.s) in the J cells (Fig. 2b, c, d) and VD2, VD3 (not shown) and a biphasic response (excitation followed by prolonged inhibition) in G group (Fig. 2i, j).

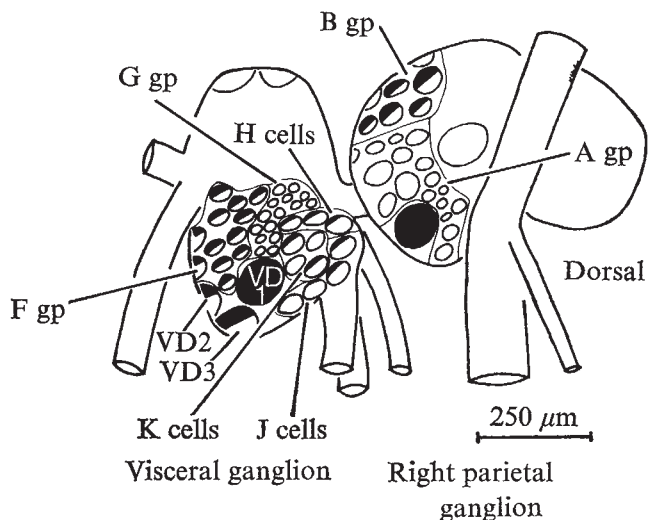


Fig. 1 Diagram of the visceral and right parietal ganglia of *Lymnaea* from the dorsal aspect. The cell body positions of several giant cells and cell clusters are indicated. Most of the neurone somata are orange in colour (unshaded), but some are pale (part shaded) and others white (filled), probably due to the light-scattering properties of small granules present in the cytoplasm. The H, J and K cells are difficult to separate from one another by appearance alone and do not necessarily lie in discrete clusters, but G group is a distinct cluster of small orange cells of 30–50 μ m soma diameter. The cell bodies of A group are of variable size, but have similar properties to one another. The somata of the giant cells VD2 and VD3 (visceral dorsal cells 2 and 3) usually lie on the dorsal surface, but may sometimes be found ventrally along with the soma of VD1. The cell bodies of F group and B group spread on to the ventral surfaces of the visceral and right parietal ganglion respectively. Neither they nor VD1 receive inputs from the RPGN, but they share some common inputs with it and with the cells it innervates.

Several lines of evidence suggest that these connections are monosynaptic:

- (1) One postsynaptic potential per spike could be evoked up to the maximum frequency of firing of the RPGN (about 10 Hz) (Fig. 2a, b).
- (2) The delay between the onset of the spike in the RPGN and the start of the postsynaptic response was constant for a particular neurone (20–25 ms for e.p.s.p.s, Fig. 2a and b) even after treatment in a solution containing five times (20 mM) the usual Ca²⁺ concentration (Fig. 2f, g, h). The delay was more difficult to determine for i.p.s.p.s because of their slow rise time.
- (3) Bathing the preparation for up to 2 h in this high Ca²⁺ saline failed to block excitatory (Fig. 2e, f, h) inhibitory (Fig. 2g) or biphasic effects. High Ca²⁺ saline would be expected to raise the threshold of any interneurone lying between the RPGN and a follower cell, thus causing synaptic block⁷.
- (4) Ionophoretic injection of tetraethylammonium (TEA) into the RPGN via a recording electrode containing 1 M TEA bromide resulted in a prolonged action potential and a corresponding increase in the duration of the postsynaptic response (shown for a G group biphasic response in Fig. 2i, j). We presume that injected TEA has the effect of blocking the delayed increase in K⁺ conductance during the action potential⁸, thereby prolonging the release of transmitter, and increasing the duration of the postsynaptic response in the case of a monosynaptic connection. On the basis of this evidence it seems reasonable to summarise the connections of the RPGN with its follower cells as shown in Fig. 2k. Further inputs common to the RPGN and its follower cells are summarised elsewhere⁶.

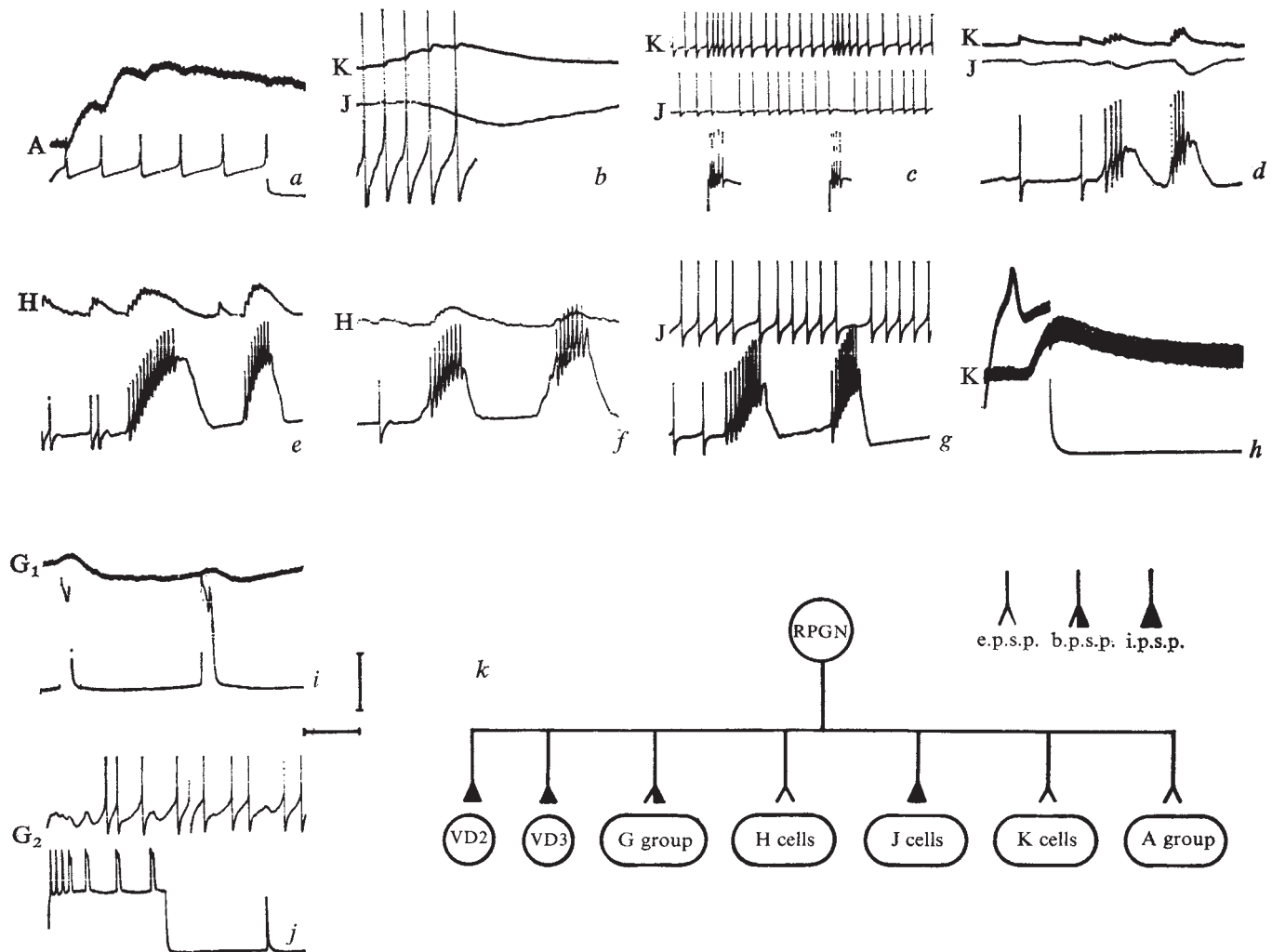


Fig. 2 Monosynaptic connections between the RPGN and its follower cells. The RPGN is on the lower trace in all cases. Other traces are from follower cells of the RPGN labelled with capital letters in accordance with Figs 1 and 2k. Excitatory inputs are received by the cells of A group (a), the K cells (b, c, d) and H cells (e), whilst i.p.s.ps are received by the J cells (b, c, d) and biphasic p.s.ps (b.p.s.ps) are found in the cells of G group (i, j). To demonstrate the 1:1 relationship and constant latency between spike and p.s.p. it is necessary to hyperpolarise the follower cells (a, b, d, e) by 5–15 mV since at the follower cells' normal firing level these effects cannot be clearly seen (c). The RPGN follower cell connections are not blocked by high Ca^{2+} saline (f, g, h). f, The same H cell as in (e) shown after soaking the preparation for 17 min in high Ca^{2+} saline. The e.p.s.ps are diminished in amplitude since increased Ca^{2+} levels cause a depolarisation of the G cells rather than a hyperpolarisation. cell RPGN spikes are increased in amplitude since they are partially Ca^{2+} dependent. g, High Ca^{2+} saline fails to block inhibition of a J cell by the RPGN; h, 20 repetitions of a spike in the RPGN, evoked by a 100-ms pulse at 0.5 Hz, cause a stable e.p.s.p. in a K cell after immersion in high Ca^{2+} saline for 15 min. The effects of TEA on the RPGN spike and on the b.p.s.ps recorded in two different G cells from the same preparation are shown in i and j. The b.p.s.ps are considerably enhanced and have durations in excess of 1.2 s compared with control durations of 0.5–0.7 s. In normal conditions the initial excitatory phase is usually very small or even suppressed. i, G_1 , 310 min after the start of iontophoretic injection of the RPGN. j, G_2 , 330 min after the start of TEA injection (0.5-s pulses at 1 Hz). k, Summary diagram of the monosynaptic connections of the RPGN. Time calibrations: a, 0.4 s; b, 0.8 s; c, d, 8 s; e, f, 2 s; g, 4 s; h, 0.1 s; i, 1 s; j, 4 s. Voltage calibrations: a, upper 4 mV, lower 100 mV; b, all traces 20 mV; c, all traces 80 mV; d, upper 20 mV, middle and lower 40 mV; e, f, upper 20 mV, lower 100 mV; g, upper 40 mV, lower 100 mV; h, upper 10 mV, lower 80 mV; i, upper 10 mV, lower 40 mV; j, upper 20 mV, lower 100 mV.

Sakharov⁹ has demonstrated that the RPGN of *Lymnaea* is catecholaminergic and Kiss¹⁰ indicated that several cells in the visceral and right parietal ganglia respond to iontophoretically applied dopamine with either depolarising or hyperpolarising shifts in membrane potential. Although the precise identity of these cells is as yet unclear they occur in similar areas of the ganglia to those described here and presumably correspond to some of the cells we describe. Thus the RPGN is most probably dopaminergic and therefore homologous with another multi-action neurone making widespread monosynaptic connections, the giant dopamine-containing cell (GDC) of *Planorbis corneus*³. The brain of *Planorbis* (a sinistral pulmonate) is a mirror image of that of *Lymnaea*. Thus the GDC lies on the left pedal ganglion and its follower cells lie on the visceral and left parietal ganglia. Unfortunately, none of the follower cells in *Planorbis* has been identified and it is not yet possible to say

whether they correspond with the identified neurones we have described in *Lymnaea*.

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Spontaneous release of transmitter from 'repressed' nerve terminals in axolotl muscle

DENERVATION of the limb of a lower vertebrate is followed by an ordered reinnervation, so that the normal pattern of muscle use is restored¹. Higher vertebrates, however, seem to be much less selective in their reinnervation². There is no absolute restriction on inappropriate reinnervation in lower vertebrates³; the return of normal function is due, at least in part, to multiple reinnervation of muscle fibres by different nerves, followed by a repression of function of less appropriate contacts⁴. Yip and Dennis⁵ have shown that the repression of function in inappropriate nerve-muscle junctions in the newt, *Notophthalmus viridescens*, involves a reduction in the number of quanta of synaptic transmitter released by each nerve impulse. If the initiating event in this process is reduction or loss of the ability of inappropriate nerve terminals to release transmitter^{6,7}, there should be evidence of the continued presence of these terminals even after transmission from the inappropriate nerve has been repressed. In this paper we present such evidence as a result of our studies in the axolotl.

Sectioning the anterior nerve to the supracoracoideus muscle of axolotls (*Ambystoma mexicanum*) induces the posterior nerve to sprout and innervate muscle fibres normally activated by the anterior nerve. This inappropriate innervation is subsequently repressed when the anterior nerve regenerates and reinnervates its normal territory⁸ (Fig. 1). In our experiments, the anterior nerve was sectioned initially and then again, 20–100 d after the first cut, so as to cause the newly restored anterior nerve terminals to degenerate once again. Nerve-muscle transmission from anterior nerve terminals failed about 24–30 h (December–January) or 48–54 h (May–June) after this second section (similar seasonal differences in the time of survival of axotomised nerve terminals occur in frogs^{9,10}). Shortly after this failure, intracellular recording revealed low frequency miniature endplate potentials (m.e.p.ps) in regions of the muscle where repressed nerve terminals were anticipated to exist (Fig. 1d), and not elsewhere (Fig. 2). These m.e.p.ps were not seen if both anterior and posterior nerves were sectioned. (Thirty-six cells were examined in two muscles. Muscles from the contralateral limbs were given the same initial operation but the posterior nerves were left intact in the second operation. Eight of 24 cells in the contralateral muscles had the low frequency m.e.p.ps.) Stimulation of the intact posterior nerve evoked no responses in muscle fibres exhibiting these low frequency m.e.p.ps.

This population of m.e.p.ps had properties distinguishing them from those recorded at degenerating terminals, those seen at developing nerve-muscle junctions at the stage before nerve-muscle transmission has developed¹⁰, and also from those caused by ACh release from denervated Schwann cells a few days after nerve-muscle transmission has failed¹¹. Degenerating anterior nerve terminals continued to release m.e.p.ps for 1–4 h after nerve-evoked transmitter release had failed and they occurred at multiple sites along the length of each muscle fibre, whereas the low frequency m.e.p.ps which remained after the degenerating terminals had ceased to release transmitter usually occurred at a single site near the distal end of a muscle fibre; their further characteristics are listed in Table 1.

Lanthanum ions increase m.e.p.p. frequency at normal and

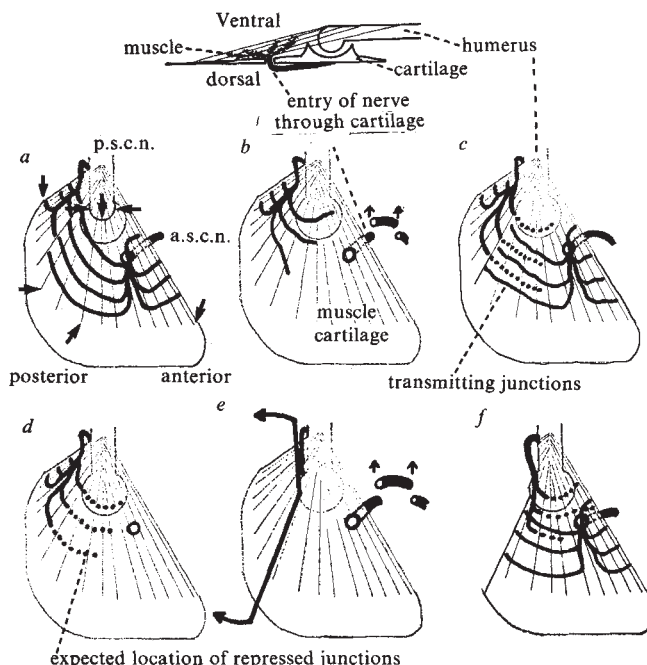


Fig. 1 Experimental scheme to locate repressed nerve-muscle junctions. Top, transverse section view through supracoracoideus muscle and cartilage; a–f, ventral views. The supracoracoideus muscle in the shoulder girdle of the axolotl has two separate zones of innervation (a). Cutting the anterior nerve (a.s.c.n.) provokes the posterior nerve (p.s.c.n.) to extend its transmission zone into more anterior regions of the muscle (b). Later, (c), the anterior nerve regenerates and resumes control of the large anterior zone of the muscle, and transmission to this zone by the posterior nerve (dotted lines) is repressed⁸. We cut the anterior nerve a second time, and shortly after its degenerating terminals had ceased to release transmitter, explored the muscle for evidence of repressed nerve-muscle junctions (d). In a second series of experiments (e, f) the whole posterior zone of the muscle was removed at the time of anterior nerve section so that any contacts between the posterior nerve and the muscle seen at a later time could be judged inappropriate. Supracoracoideus muscle fibres are multiply innervated by axons from one or the other nerve, and respond to nerve stimulation with action potentials. For microelectrode recording much of the cartilage was removed and the muscle approached from the dorsal (cartilage) side. The reference points marked by arrows (a) were mapped for each muscle using a 20× water immersion objective and the microscope stage micrometer so that relative positions of recording sites in muscles of different sizes could be compared by transferring these coordinates to a common schematic. The axolotls used in this work were all from the same mating and hatched in September 1975.

developing nerve-muscle junctions^{10,12}. In control experiments, using normal muscles, 0.5 mM LaCl_3 caused a 10–100-fold increase in m.e.p.p. frequency within 30 s of its addition to the perfusion system. Most applications to experimental preparations were of 2 mM LaCl_3 , and in about half 10 mM HEPES buffer (pH 7.2) was added to avoid problems of excess acidity or precipitation of La^{3+} . Twenty-three muscle fibres with low frequency m.e.p.ps seen shortly after failure of anterior nerve transmission were examined for 2 min or more during perfusion with La^{3+} Ringer with a resolution of recording of 0.2 mV or better. In 19 fibres there was no change in m.e.p.p. frequency (less than twofold increase or decrease) during application of the La^{3+} . Two fibres responded with a large increase in m.e.p.p. frequency. In two more there was a slowly developing increase after an initial latent period; for example, control frequency 5 per 30 s; La^{3+} induced, 7, 6, 10, 30, and 51 per 30 s respectively.

Depolarisation of normal or developing nerve-muscle junctions by adding KCl to the bathing medium causes a prompt and maintained increase in m.e.p.p. frequency¹⁰. In control experiments with normal muscles, KCl concentrations of 15 or 17.5 mM caused an increase in m.e.p.p. frequency at least tenfold within seconds of its addition to the bath, whereas muscle fibres were not so depolarised that m.e.p.ps were

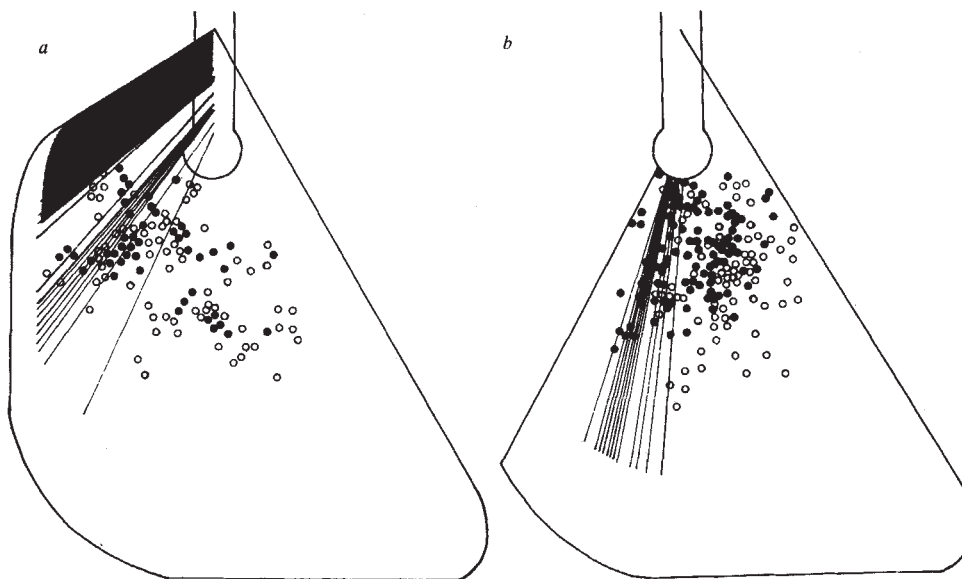


Fig. 2a Posterior nerve innervation zone and location of low frequency m.e.p.ps in supracoracoideus muscles following expansion and retraction of the posterior nerve innervation zone. Records from both left and right muscles from animals ranging in length from 5 to 13 cm have been placed on a common diagram using the reference points illustrated in Fig. 1a. ●, Fibres with low frequency m.e.p.ps and no response to posterior nerve stimulation; ○, fibres with no m.e.p.ps over a period of at least 2 min stable recording with resolution 0.2 mV or better. Shaded area, fibres contracting in response to posterior nerve stimulation in normal (control) muscles; /, borders of posterior nerve-induced contraction in individual experimental muscles. The diagram summarises results from 16 muscles examined 20–52 d after the initial section of the anterior nerve (in seven muscles the nerve was resectioned 3 weeks after the initial operation, and the muscles examined 2–3 weeks later). The anterior nerve was again sectioned 24–96 h before recording from all of the muscles. Degenerating anterior nerve terminals ceased to release transmitter 24–30 h after nerve section in these

animals. Low frequency m.e.p.ps with the special properties described in the text could be identified only anterior to the border of posterior nerve-muscle transmission. This border had retracted to within its normal limits in less than half the muscles examined in this series. These experiments were carried out during January and February 1976. *b*, Nerve-muscle transmission and low frequency m.e.p.ps in preparations with the posterior portion of muscle removed at the time of anterior nerve section. Symbols as in *a*. This is a summary of results from 16 preparations examined 3 months after the initial operation, and 29–120 h after second section of the anterior nerve. These animals ranged in size from 14 to 19 cm, so anterior nerve regeneration generally took longer than in the muscles illustrated in *a*. The experiments were carried out during May and June 1976. In these experiments all muscle fibres became reinnervated by the anterior nerve, but 12 of 16 muscles retained a zone of posterior nerve innervation so that dually innervated muscle fibres occurred near the posterior border of many muscles.

difficult to see. We examined 17 junctions with low frequency m.e.p.ps seen shortly after failure of anterior nerve transmission. In 14 of these there was no change in m.e.p.p. frequency (less than twofold increase or decrease) during at least 1 min of muscle depolarisation caused by perfusion with high K^+ Ringer (the muscle cell depolarisation served to monitor the effective presence of the K^+). In two fibres there was a prompt and large increase in m.e.p.p. frequency and in one fibre it began to increase 2 min after addition of the K^+ (control frequency 4 min^{-1} ; K^+ , 4 min^{-1} , 7 min^{-1} , 25 min^{-1}).

Slowly developing increases in m.e.p.p. frequency in response to La^{3+} or K^+ perfusion were more commonly seen when more time elapsed after anterior nerve section. For example, in muscles examined 5 d after nerve section five fibres treated with La^{3+} showed no change in frequency during the first 2 min of perfusion, but increased an average of six times during the third minute. Similarly, three fibres treated with high K^+ showed a sixfold increase in m.e.p.p. frequency during the third minute. Also, at this time, a few fibres were found where posterior nerve stimulation intermittently evoked small e.p.ps. Genat and Mark⁸ found that transmission at repressed junctions in axolotl supracoracoideus muscle began to return 7 d after section of the regenerated anterior nerve. The slight restoration of sensitivity of m.e.p.p. frequency to La^{3+} or K^+ seen 5 d

after anterior nerve section may mark a return towards normal function.

Increased tonicity raises m.e.p.p. frequency at normal and developing nerve-muscle junctions^{13,14}. Twenty-one fibres with low frequency m.e.p.ps were tested by application of Ringer solution made hypertonic by addition of 55 mM Na_2SO_4 . In 20 of these fibres m.e.p.p. frequency increased at least fivefold. Seven fibres were tested with La^{3+} and K^+ in addition; mean m.e.p.p. frequencies were: control, 10 min^{-1} ; K^+ , 14 min^{-1} ; La^{3+} , 8 min^{-1} ; hypertonic, 145 min^{-1} .

Botulinum toxin blocks nerve-evoked transmitter release and greatly reduces spontaneous m.e.p.p. frequency without affecting synaptic structure or propagation of the presynaptic action potential¹⁵. We treated normal axolotl muscles with large doses of type D botulinum toxin and found that the resulting low frequency m.e.p.ps had properties similar to those seen following repression of inappropriate nerve terminals. Seven botulinised fibres examined in normal Ringer had a mean m.e.p.p. frequency ($\pm$ s.e. of the mean) of $0.10 \pm 0.04 \text{ min}^{-1}$; in hypertonic Ringer $9.9 \pm 5.8 \text{ min}^{-1}$; and in KCl Ringer $0.14 \pm 0.04 \text{ min}^{-1}$.

The low frequency m.e.p.ps seen in the experimental muscles resembled those released from Schwann cells at denervated endplates in that their frequency was unaffected by treatment

Table 1 Properties of miniature endplate potentials at normal, developing, degenerating, denervated and repressed nerve-muscle junctions

Agent	Junction				
	Normal	Developing	Degenerating	Denervated (Schwann cells)	Repressed
Nerve stimulation	e.p.p. or a.p.	No effect	Variable	No effect	No effect
Nerve section	Abolished	Abolished	—	No effect	Abolished
Frequency (min^{-1})	10–60	0.2–60	0.2 to very high, in bursts	0.2–10	0.2–10
La^{3+}	Increased frequency	Increased frequency	Increased frequency	No effect	No effect
K^+	Increased frequency	Increased frequency	Increased frequency	No effect	No effect
Hypertonic solutions	Increased frequency	Increased frequency	Not tested	No effect	Increased frequency

with La^{3+} ions or by raised K^+ in the bathing medium¹⁰. Unlike Schwann-released m.e.p.ps, they were very sensitive to increased tonicity of the bathing medium, and no 'silent period'^{10,11} elapsed before they could be seen (we saw no Schwann m.e.p.ps in axolotl muscles kept fully denervated for up to 42 d, in spite of recording close to old endplates).

The unique properties of these m.e.p.ps (Table 1), together with their territorial distribution in experimental muscles (Fig. 2), and their dependence on the integrity of the posterior nerve, distinguish them from other classes of m.e.p.ps, and we suggest that they are released from nerve terminals whose normal function has been repressed (and possibly also from developing inappropriate nerve terminals whose maturation has been arrested).

On the basis of our observations we propose that repression of transmission at inappropriate junctions in axolotl muscle is due to a change in or loss of the Ca^{2+} -dependent mechanism^{16,17} involved in transmitter release from nerve terminals. At normal and developing endplates La^{3+} ions seem to affect transmitter release by activating this Ca^{2+} -dependent process¹², and the effect of K^+ ions on transmitter release requires the presence of Ca^{2+} (ref. 14). On the other hand, the effect of hypertonic solutions is independent of the presence of Ca^{2+} (ref. 14). Also, the properties of the 'repressed' m.e.p.ps were mimicked by botulinising normal nerve terminals, and it has recently been shown that m.e.p.p. frequency in botulinised nerve-muscle preparations is returned to normal by treating them with a calcium ionophore in the presence of high external Ca^{2+} (ref. 18). We do not know how long functionally repressed junctions may remain physically present in a muscle.

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Are transmitter release statistics meaningful?

A BASIC premise of synaptic physiology is that neurotransmitter is released from motor nerve terminals in multi-molecular packets called quanta^{1,2}. The rate of release of quanta is low at rest, but when an action potential invades the nerve terminal there is a large increase in the mean rate of release leading to the generation of an

endplate potential. It has been found that the distribution of the number of quanta released by an action potential is accurately described by a binomial distribution³⁻⁸. The assumption which underlies the binomial process is that n independent units (quanta) each have the same probability of success (release), p , on a single trial (stimulation). The parameters n and p can be estimated from the mean and variance of the binomially distributed variable. Although it is not clear what the physical correlates of n and p might be, it has been suggested that n may refer to the number of releasable quanta¹, or to the number of release sites which can be activated on stimulation⁶. The changes in the apparent values of n and p which occur during synaptic processes such as facilitation^{6,8} and depression⁷, have been interpreted as providing evidence for particular mechanisms which may underly those processes.

Recent studies have shown that the probability of quantal release, either spontaneous or evoked, is increased after a spontaneous release^{10,11}. This suggests that in normal conditions p and/or n may vary with time. The bursts of spontaneous release produced by the addition of brown widow spider venom occur at localised areas of the nerve terminal¹², suggesting that spatial variations in p or n may exist. Studies of the changes in the apparent values of n and p which occur during neuromuscular depression have been interpreted as indicating that the release probability may be different for different quanta⁷. These experimental findings have led us to investigate the implications of a distribution of quantal release probabilities. As a result of these studies we believe that the application of the binomial distribution to transmitter release may not be appropriate.

A simple example can easily demonstrate the effects of a distributed probability of release. Consider an experiment in which the mean quantal content, m , is equal to 16 and the variance, σ_m^2 , is equal to 8. Since $m = np$ and $\sigma_m^2 = np(1-p)$ for a binomial process it is easy to show that $p = 0.5$ and $n = 32$. An identical mean output and variance could, however, be obtained from a population of 50 quanta, 10 with a probability of 0.8 and the rest with a probability of 0.2. Simple examples like this show that a distributed probability can cause large errors in the estimation of binomial parameters from the mean and variance of the quantal contents. Before this conclusion can be accepted however, it is necessary to demonstrate that the shape of the distribution of quantal contents cannot be distinguished from the binomial prediction by goodness-of-fit statistics. A computer simulation was necessary to generate and compare the actual and the predicted distributions. A number of different probability distributions were investigated, produced by defining a new variable $z = \log_e p/(1-p)$ and generating a normal distribution of z with mean z and variance σ_z^2 . The z values were then converted back into p values. The main advantage of this approach is that a family of distributions of p can be generated which have no quanta at $p = 0$ or $p = 1$. A trial was then run by generating rectangularly distributed random numbers and comparing them in turn with the elements of the p distribution. If the value of p for a quantum was more than the random number then that quantum was counted as being released in that trial. A number of trials were run until a smooth distribution of quantal contents was obtained. Figure 1 illustrates the results of one simulation in which most of the 100 quanta had very low probabilities of release. One hundred trials were simulated. There is no significant difference between the simulated distribution of quantal contents and that predicted for a binomial process ($\chi^2 = 4.98$ with 5 d.f., $P > 0.25$). It is interesting to note that only 1% of the quanta have probabilities of release equal to, or greater than the estimated mean probability of release.

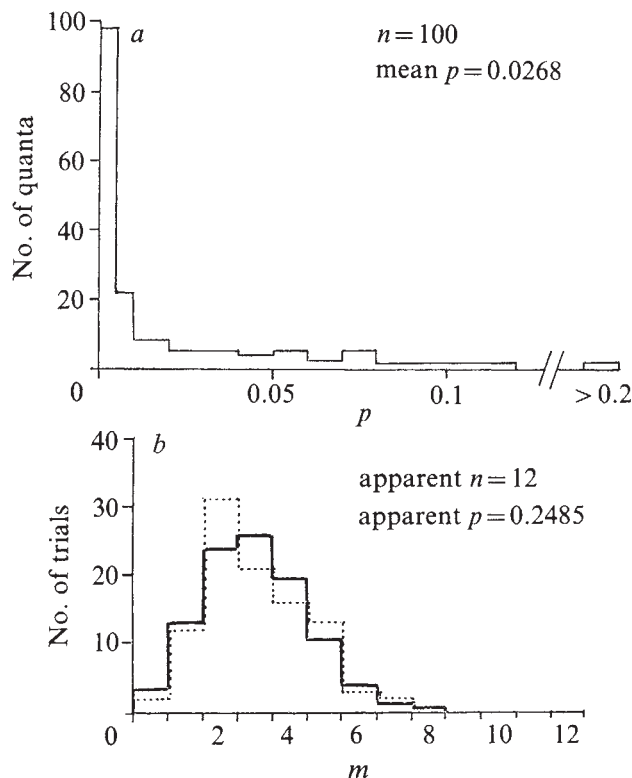


Fig. 1 The results of the simulation. *a*, Distribution of the probability of release for 100 quanta ($z = -5$, $\sigma_z^2 = 2$). *b*, Simulated distribution of quantal contents (---) and the binomial prediction (—) with parameters derived from the mean and variance of the simulated distribution.

Is it possible to establish that the quantal probabilities are not equal? In general, this is not possible for moderately sized data sets, but in special circumstances it should be possible to record quantal contents on single trials that exceed the estimated value of n . Such a result is strong evidence against the validity of binomial statistics, and has been observed at the crayfish neuromuscular junction⁹.

The results are clear. Should the release probability for individual quanta be distributed about a mean value, instead of having no variance, then the application of binomial analysis is unjustified even if the considerable practical difficulties involved in the estimation of quantal content can be overcome. Thus, in order to assess the statistics of transmitter release, it is essential to estimate the distribution of the release probabilities. Without this information it is difficult to see how a meaningful analysis can be achieved.

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Replication of EBV in epithelial cells during infectious mononucleosis

INFECTIOUS mononucleosis is caused by primary infection with the Epstein-Barr virus (EBV)^{1,2}. This virus is generally held to be trophic for B lymphocytes^{3–6}, but only latently infected cells expressing a nuclear antigen (EBNA)⁷ have been found among circulating lymphocytes during infectious mononucleosis⁸. In spite of this, EBV is present in the oropharyngeal secretions of most patients with infectious mononucleosis, and virus continues to be shed in many normal persons for extended periods^{9,10}. The source of this virus, a cell type which freely permits replication of EBV, has never been identified. An intriguing link has recently been advanced between EBV and the malignant epithelial cells of undifferentiated carcinoma of the nasopharynx. Patients with this malignancy maintain high titres of antibodies to the virus¹¹. The tumour itself, which consists of malignant epithelial cells with many infiltrating lymphocytes, contains the EBV genome¹². The EBV genetic material is in the malignant epithelial cells^{13,14}, however, and not in the infiltrating lymphocytes which are predominantly T cells. Although superinfection of nasopharyngeal carcinoma cells with EBV leads to the expression of specific early viral antigens¹⁵, these cells, like B lymphocytes, are only latently infected *in vivo*. The primary cell type infected by EBV probably occupies a pivotal position in the pathogenesis of both infectious mononucleosis and nasopharyngeal carcinoma. We report here evidence directly from human material that EBV replicates in epithelial cells of the oropharynx.

Patients with infectious mononucleosis were selected on the basis of a compatible clinical syndrome, a positive 'Monospot test' (Ortho-Diagnostics, Inc.) and high antibody titres to EBV early antigen (EA) and EBV viral

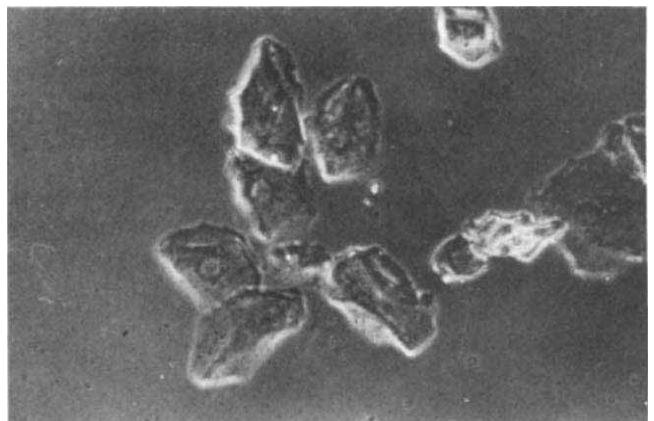


Fig. 1 cRNA-DNA *in situ* cytohybridisation of oropharyngeal epithelial cells from an EBV-antibody negative donor. 8×10^6 c.p.m. ³H-cRNA (specific activity 1×10^7 c.p.m. μg^{-1}) was hybridised under coverslips as described^{19,20} for 22 h at 66 °C. Slides were then washed four times in NaCl 0.3 M, sodium citrate 0.03 M (2X-SSC), treated with RNase 20 $\mu\text{g ml}^{-1}$ for 1 h at 37 °C, washed again four times with 2X-SSC, dipped twice in 70% ethanol and then twice in 95% ethanol. Kodak Nuclear Tract NTB2 emulsion was used for autoradiography; exposure was for six weeks at -20 °C. In this specimen, which did not contain EBV, a few scattered grains are seen over each of several epithelial cells. (Unstained, phase contrast, $\times 475$.)

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capsid antigen (VCA) (ref. 16). Throat washings obtained by gargling with phosphate-buffered saline solution (PBS) were centrifuged at low speed; the supernatant fluids were passed through a 0.45- μ m filter and stored at -70°C in 10% foetal calf serum. Specimens were tested for EBV by an assay based on stimulation of DNA synthesis in human umbilical cord lymphocyte cultures as described previously¹⁷. Those throat washings which gave maximal stimulation of thymidine incorporation at 37 d of culture were selected for *in situ* cytohybridisation with EBV-specific complementary RNA (cRNA). Previous efforts to demonstrate EBV-infected cells in oropharyngeal material by indirect or anticomplement immunofluorescence techniques had failed due to intense nonspecific fluorescence.

Centrifugation of each throat washing yielded readily identifiable epithelial cells. The cell pellets were washed twice in PBS, resuspended in ice-chilled methanol-glacial acetic acid (3:1) for 5 min and fixed to glass slides for *in situ* cytohybridisation. EBV DNA was purified¹⁸ and EBV-specific ³H-labelled cRNA was synthesised and hybridised as described previously^{19,20} to cell smears prepared from specimens known to contain EBV. Cells from a donor without serological evidence of previous EBV infection were also tested. After extensive washing and RNase treatment, hybridised cRNA was demonstrated by autoradiography¹⁹.

Cells from the EBV-negative donor demonstrated a low background of grains diffusely scattered over numerous epithelial cells (Fig. 1). Specimens known to contain transforming virus presented an entirely different picture. The number of grains over almost all epithelial cells was higher than in the control preparations (Fig. 2a, b). Much more striking, however, was the appearance of dense accumulations of grains over a minority of epithelial cells, often seen in close proximity to other cells which hybridised far less of the EBV-specific cRNA (Fig. 2a). The density of grains was uniform over most of these EBV-containing cells, but occasionally there were denser accumulations of grains over the nucleus (Fig. 2b).

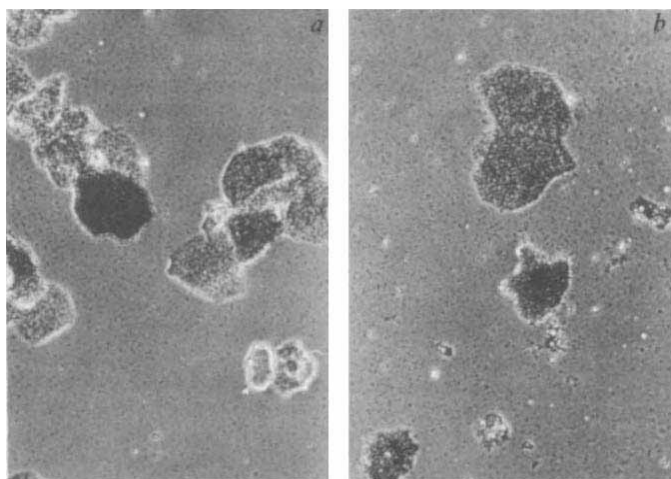


Fig. 2 a, Cytohybridisation of oropharyngeal epithelial cells from a patient with infectious mononucleosis and mild pharyngitis. Anti-EA was 1:160 and anti-VCA was 1:160; supernatant fluid from this throat washing resulted in a sevenfold stimulation of thymidine incorporation by cord lymphocytes after 37 d of culture. Note one cell over which there is a dense accumulation of grains while adjacent cells have hybridised relatively little EBV-specific cRNA. b, Cytohybridisation of oropharyngeal cells from a second patient with infectious mononucleosis and moderately severe tonsillopharyngitis. Anti-EA was 1:40 and anti-VCA was 1:160; supernatant fluid resulted in fivefold stimulation of cord lymphocytes. Several EBV-infected cells are present; in one, grains are concentrated over the cell nucleus. (a, b, Unstained, phase contrast, $\times 674$.)

The number of grains over the most positive of these EBV-infected cells was equivalent to that seen over P3HR-1 cells that replicate EBV DNA and produce virus (Fig. 3). As the sensitivity of *in situ* cytohybridisation is rather low (about 60 EBV genomes per cell)²⁰, the results indicate a very high number of EBV genomes per positive epithelial cell. This itself constitutes strong evidence not only for infection with EBV but also for *in vivo* replication of the virus.

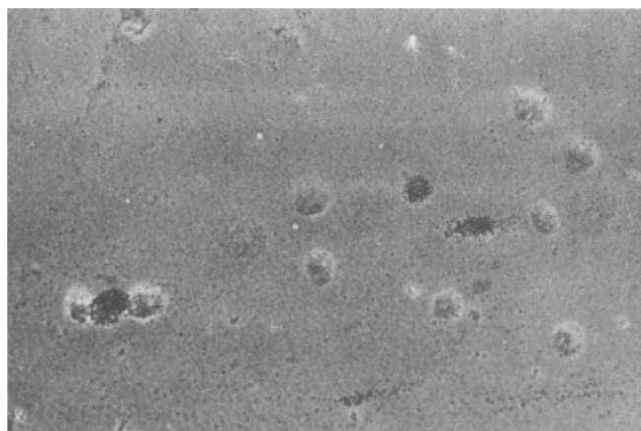


Fig. 3 A virus-producing lymphoid cell line (P3HR-1), derived from African Burkitt's lymphoma, which was hybridised at the same time as the oropharyngeal cells. Four EBV-producing cells can be seen, whereas other, latently infected lymphoid cells did not hybridise appreciable amounts of cRNA. The density of grains over the virus-producing cells is equivalent to that over EBV-infected epithelial cells in Fig. 2a and b. (Unstained, phase contrast, $\times 674$.)

Since there is virus in the throat, virus might adsorb on to epithelial cells. This would give fewer grains, however, not localised on the nucleus and not spare adjacent cells. To control for this possibility, normal epithelial cells were exposed to a filtrate of a mononucleosis throat washing before cytohybridisation. This exposure produced little adsorption of virus as disclosed by hybridisation, even though the filtrate contained transforming virus and in spite of the presence of EBV-infected epithelial cells in the original mononucleosis specimen (Fig. 4).

When stained with methylene blue-basic fuchsin, the EBV-infected cells showed the small centrally placed nuclei and abundant cytoplasm typical of epithelial cells. There was no obvious cytopathic effect, but morphology following NaOH denaturation was less than optimally preserved.

Our results indicate that EBV infects and replicates within epithelial cells during the course of infectious mononucleosis. This observation answers several longstanding questions. It suggests that the epithelial cell is the source of the virus found in throat washings, consistent with the observation that virus shedding can occasionally be localised in the anterior oropharynx where lymphoid tissue is scant, but epithelium abundant, rather than the posterior pharynx and tonsillar areas¹⁰. Infection of the oropharyngeal epithelium may also account for the intense pharyngitis often seen in infectious mononucleosis. Virus presumably reaches the tonsils by way of the epithelium, where infection and transformation of the B lymphocyte occurs²¹. The transformed B lymphocyte then evokes a cytotoxic cellular response, characterised by the atypical lymphocyte which may by itself be largely responsible for the systemic manifestations of the disease^{22,23}.

It has been difficult to reconcile the presumed lymphotropic nature of EBV with the presence of EBV DNA in the malignant epithelial cells of nasopharyngeal carcinoma. Although the lymphocyte has long been considered

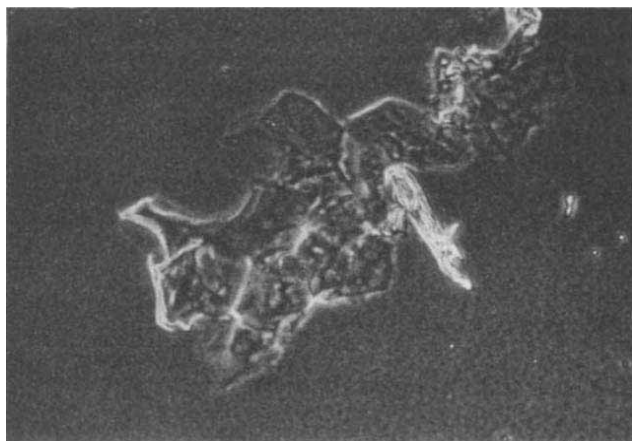


Fig. 4 *In situ* cytohybridisation of epithelial cells from the throat of a patient without antibodies to EBV after adsorption with supernatant fluid from a throat washing that contained transforming virus. Cells collected from the normal donor were washed twice in PBS and resuspended in the supernatant filtrate from the mononucleosis patient described in Fig. 2a. The normal cells were allowed to adsorb virus for 2.5 h at 4 °C, washed twice in PBS, fixed in methanol-acetic acid and processed for cytohybridisation as described in Fig. 1. There are only scattered grains over the cluster of epithelial cells. (Unstained phase contrast, $\times 674$.)

the primary cell infected with EBV, it may well be that the primary cell is instead the epithelial cell. No other cell type has been shown to support EBV replication *in vivo*. Epithelial cell infection may be predominantly lytic and productive. Occasionally, however, perhaps influenced by genetic factors, there may be arrest of viral DNA replication, formation of EBV DNA episomes²⁴, and transformation of the epithelial cell. Such a transformed cell may be the progenitor of nasopharyngeal carcinoma.

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5'-Terminal nucleotide sequences of polio virus polyribosomal RNA and virion RNA are identical

POLIO virus is a positive strand virus; it has a mRNA of the same 5' to 3' polarity and size as its virion RNA (ref. 1). It also has a protein covalently linked to its 5'-terminal end forming a 5'-terminus of protein-pU-U-A-A-A-A-C-A-Gp (refs 2, 3). This contrasts with other viral RNAs that either have di- or triphosphate 5'-termini (negative strand RNAs)^{4–6} or m⁷G^{ppp}N(m)p . . . termini (most positive strand RNAs)^{7,8}. The 5'-linked protein on poliovirus RNA is also found on at least two-thirds and perhaps all of the nascent chains of the replicative intermediate isolated from infected cells³. This suggests that the protein is linked to the RNA at a very early stage of replication, perhaps as a primer during the initiation of RNA synthesis^{2,3}. The 5'-terminus of polio virus polyribosomal RNA lacks the protein and instead terminates in pUp . . . (refs 9, 10). Thus, it is possible that the protein initially present on the newly synthesised RNA chains is cleaved from that fraction of the RNA molecules destined to become mRNA. To determine the nucleotide sequence relationship between the polio virus RNAs containing and lacking the protein, we have isolated and sequenced the 5'-terminus of poliovirus polyribosomal RNA. We show here that its nucleotide sequence is identical to that of the 5'-terminus of virion RNA.

To identify the 5'-terminal oligonucleotide of poliovirus polyribosomal RNA we used the characteristic that distinguishes it from all other oligonucleotides in the RNA, the presence of a pUp residue. The total mixture of RNase T₁ oligonucleotides was fractionated by ionophoresis on cellulose acetate in the first dimension and homochromatography on polyethyleneimine (PEI)-cellulose plates in the second dimension^{11,12} (Fig. 1a). To find the 5'-oligonucleotide, regions of the fingerprint were scraped from the PEI-cellulose plate and digested with RNases T₁, T₂ and A. The products were separated by paper electrophoresis at pH 3.5 (ref. 11). Only one spot on the fingerprint gave rise to pUp which was identified by its characteristic mobility just slower than orthophosphate³. An arrow indicates the pUp-containing spot in Fig. 1a. The rest of the regions analysed gave rise only to 3'-monophosphates. The identification of the pUp-containing oligonucleotide as the 5'-terminus of polyribosomal RNA is supported by its absence from the fingerprint of virion RNA (Fig. 1b, arrow). For virion RNA, the protein-linked 5'-terminal T₁ oligonucleotide would have been excluded from the second dimension because of its slow mobility in the first dimension³.

The intensity of the pUp-containing spot was weaker than that of the neighbouring spots suggesting that it either was present in a submolar amount or that it contained fewer phosphates than the adjacent oligonucleotides. The two oligonucleotides to the right of the pUp-containing spot were determined to be about 18 and 20 nucleotides long. This was based on a quantitation of the RNase P₁ digestion products relative to the radioactivity in the

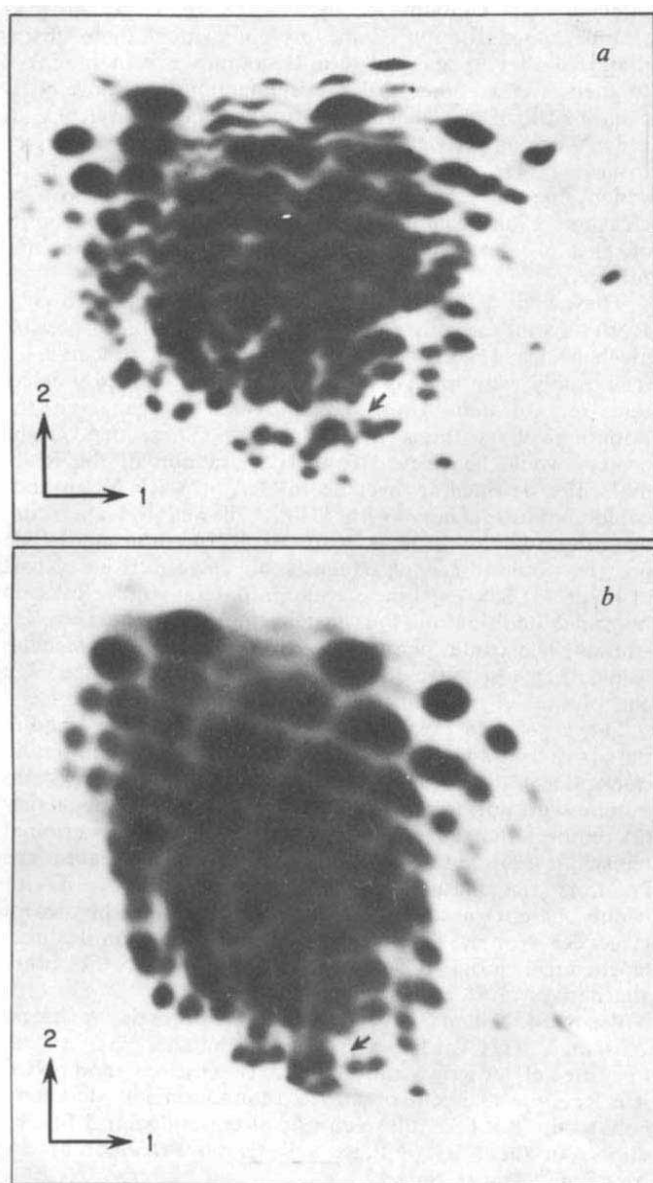


Fig. 1 Two-dimensional separation by electrophoresis and thin-layer chromatography of RNase T_1 oligonucleotides derived from polio virus polyribosomal and virion RNA. Polio virus-specific RNA labelled with ^{32}P -orthophosphate (New England Nuclear) was prepared by infecting actinomycin D-treated HeLa cells in suspension with polio virus type 1 as described previously^{3,9}. RNA from purified virions labelled with ^{32}P (collected 6 h post-infection) was prepared by extraction with sodium dodecyl sulphate-phenol (SDS-phenol) and purified by sucrose gradient centrifugation as described previously¹⁷. Polio virus polyribosomal RNA was prepared from cells collected 3.5 h post-infection as described previously^{9,16}. In summary, a cytoplasmic extract was taken and the polyribosomes collected on a sucrose cushion¹⁷. The polyribosomal RNA was precipitated with 2 M LiCl in the presence of 0.5% SDS at -20°C . The precipitate was extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and the RNA was recovered by ethanol precipitation and centrifugation. The RNA was further purified by oligo(dT) cellulose chromatography and sucrose gradient centrifugation. The peak fractions of 35S RNA were pooled and the RNA was precipitated by two volumes of ethanol. Yields of between 1 and 3×10^7 c.p.m. (Cherenkov radiation) of ^{32}P -labelled polyribosomal RNA were regularly obtained from 4×10^8 cells and 60 mCi ^{32}P -orthophosphate. Purified polyribosomal and virion RNAs were digested with RNase T_1 for 30 min at 37°C in 3 μl of 10 mM Tris, pH 7.4, and 1 mM EDTA containing 10 μg of RNA and 200 U ml^{-1} of T_1 (Calbiochem). The resulting oligonucleotides were subjected in the first dimension to electrophoresis on Cellogel strips (Chemeton, Milan) at 2,500 V for 70 min at pH 3.5 (ref. 11). Separation in the second dimension was by chromatography on plastic-backed polyethyleneimine-cellulose thin-layer plates (20 $\times$ 20 cm, Brinkmann Instruments)^{11,12}. Homomixture C (ref. 11) digested for 2 min with 1 M KOH was used for the second dimension. *a*, Autoradiogram of a fingerprint of polio virus polyribosomal RNA; *b*, autoradiogram of polio virus virion RNA. In (*a*) the arrow indicates the position of the pUp-containing 5'-terminal oligonucleotide and in (*b*) the arrow indicates the absence of the spot from the virion RNA. Different batches of homomixture were used in (*a*) and (*b*).

5'-GMP. Correction for the unequal labelling of the four monophosphates was done as described previously³ and in the legend to Table 1. As shown below the length of the 5'-terminal oligonucleotide is only 9 nucleotides (10 phosphates). Apparently the additional 5'-phosphate in the terminus substantially retards the mobility of the oligonucleotide during homochromatography.

Table 1 Ribonuclease A and U_2 products derived from the 5'-terminal oligonucleotide of polio virus polyribosomal RNA

	Product	Yield*
RNase A†:	A-A-A-A-Cp	1.0
	A-Gp	1.0
	pUp	0.7
	Up!‡	0.9
RNase U_2 §:	[pUp, Up, Cp, (Ap) _n]	
	[pUp, Up, (Ap) _n]	
	A-Ap	
	C-Ap	
	Gp	
	Ap	

*The yield of RNase A products was calculated relative to A₄C. Corrections for unequal labelling of the four nucleoside monophosphates were done³. The ratios of the radioactivity of the 5' monophosphates to that of the 3' monophosphates were found to be 1.0 for CMP, 0.8 for AMP, 0.6 for GMP and 1.6 for UMP. In the case of Gp, where the nearest neighbour was unknown, a specific activity of 1.0 was used for the phosphate.

†Digestion conditions were: 30 min at 37°C in 5 μl of 20 mM Tris, pH 7.5, and 2 mM EDTA, containing 15 μg of yeast RNA (Sigma) and 7.5 μg of RNase A (Worthington).

‡All the uridine monophosphate was recovered as 2',3' cyclic UMP.

§Only the composition of the two largest RNase U_2 products is indicated because exact sequences were not determined. The U_2 digestion conditions were: 2 h at 37°C in 10 μl of 15 mM sodium acetate, pH 4.5, and 1 mM EDTA containing 0.01 U of U_2 (Calbiochem). In these conditions the U_2 products presumably contained 2',3' cyclic monophosphate termini.

Further purification of the oligonucleotide was required for sequencing purposes and was accomplished by elution of the oligonucleotide and fractionation on a two-dimensional polyacrylamide gel^{6,13}. A major component migrated in the first dimension together with the bromophenol blue dye and in the second dimension slightly faster than it (Fig. 2). A few faint spots representing contaminating oligonucleotides from the first two-dimensional fingerprint could also be observed. Only the major spot contained pUp and therefore must have been the 5'-terminal T_1 oligonucleotide. Molar yields of secondary digestion products (Table 1) established the purity of this oligonucleotide.

To determine the sequence of the oligonucleotide, it was eluted, digested with RNase A or RNase U_2 , and the products were separated by ionophoresis on DEAE-paper at pH 3.5 (ref. 11). RNase A digestion gave rise to four products, which were identified as A-A-A-A-Cp, A-Gp, pUp, and Up! on the basis of their migration relative to marker oligonucleotides and subsequent base composition analysis. All four products were present in approximately equimolar amounts (Table 1). The order of the RNase A products was established from the RNase U_2 digestion products (Table 1). The product C-Ap showed that the RNase A products A-A-A-A-Cp and A-Gp must have

been digested with RNase T_1 for 30 min at 37°C in 3 μl of 10 mM Tris, pH 7.4, and 1 mM EDTA containing 10 μg of RNA and 200 U ml^{-1} of T_1 (Calbiochem). The resulting oligonucleotides were subjected in the first dimension to electrophoresis on Cellogel strips (Chemeton, Milan) at 2,500 V for 70 min at pH 3.5 (ref. 11). Separation in the second dimension was by chromatography on plastic-backed polyethyleneimine-cellulose thin-layer plates (20 $\times$ 20 cm, Brinkmann Instruments)^{11,12}. Homomixture C (ref. 11) digested for 2 min with 1 M KOH was used for the second dimension. *a*, Autoradiogram of a fingerprint of polio virus polyribosomal RNA; *b*, autoradiogram of polio virus virion RNA. In (*a*) the arrow indicates the position of the pUp-containing 5'-terminal oligonucleotide and in (*b*) the arrow indicates the absence of the spot from the virion RNA. Different batches of homomixture were used in (*a*) and (*b*).

been linked in that order. The Up residue therefore must have been between pUp and A-A-A-A-Cp and we conclude that the sequence of the total oligonucleotide is pU-U-A-A-A-A-C-A-Gp. The sequence of the two large RNase U₂ products must have been pU-U-A-A-A-A-C-Ap and pU-U-(A_n)-Ap considering the RNase A digestion products. These products are consistent with the known specificity of RNase U₂ (ref. 11) and confirm the sequence. Thus, the nucleotide sequences of the 5'-terminal T₁ oligonucleotides from polio virus virion and polyribosomal RNA are identical, but the polyribosomal RNA lacks the protein at its 5'-end. We have also analysed the complete RNase T₂ products derived from the oligonucleotide on a two-dimensional thin-layer chromatography system¹⁴ and found no evidence for any modified bases in the oligonucleotide (data not shown).

We have described a method for purifying to homogeneity the 5'-end T₁ oligonucleotide from polio virus polyribosomal RNA. Although the oligonucleotide contains only nine bases, its 5'-phosphate causes it to migrate slowly during homochromatography through PEI-cellulose positioning it in the region of the fingerprint where it is detectable as an individual spot. To purify it sufficiently for sequencing, further fractionation was needed and the two-dimensional electrophoretic system of De Wachter and Fiers¹³ provided the needed resolution. This system fractionates mainly on the basis of base composition and length and would be expected to show less effect from the 5'-phosphate.

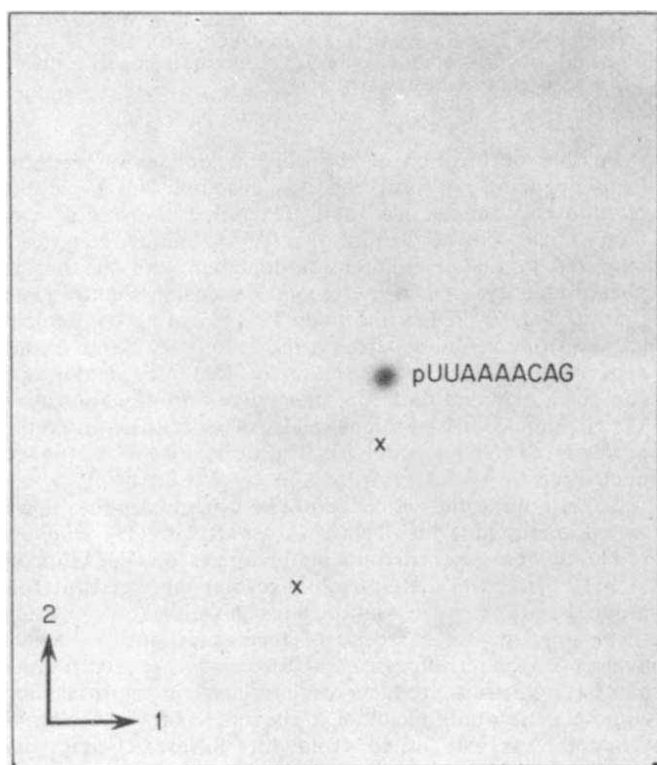


Fig. 2 Purification of the 5'-terminal oligonucleotide of polio virus polyribosomal RNA by two-dimensional gel electrophoresis. The pUp-containing T₁ oligonucleotide from the PEI-cellulose plate (Fig. 1a) was eluted with triethanolamine bicarbonate and fractionated by two-dimensional polyacrylamide gel electrophoresis as described previously^{6,13}. The first dimension (10% acrylamide, 6M urea, pH 3.5) was run until the bromophenol blue dye had migrated 20 cm. The second dimension (21.8% acrylamide in Tris-citrate buffer, pH 8.0) was run until the bromophenol blue dye had migrated 8 cm. The upper × indicates the position of the bromophenol blue dye and the lower × that of xylene cyanol. The sequence of the 5'-terminal oligonucleotide is as indicated.

The replicative intermediate isolated from polio virus-infected cells contains as an average about six growing strands¹⁵. Quantitation of the protein-pUp in these chains indicated that at least two-thirds contain a protein linked to their 5'-end³. Since mRNA is infectious³, it most likely contains all of the genetic information in virion RNA. It is therefore unlikely that the polyribosomal RNA is derived from a larger precursor molecule by any mechanism in which one or more 5'-terminal nucleotides are lost by cleavage. This conclusion is supported by the identity of the 5'-terminal nucleotide sequences in virion and mRNA.

These considerations suggest that initiation of polio virus RNA synthesis must occur by one of three possible mechanisms: (1) the 5'-terminal protein or a precursor of it, possibly with one or several covalently-linked nucleotides (e.g. protein-pU_{OH}), could act as a primer in the initiation of synthesis of all strands. Subsequently, the protein would be cleaved from that fraction of the RNA molecules destined to become mRNA. (2) RNA synthesis could initiate *de novo* with UTP, followed by rapid condensation of the protein with all RNA molecules. The protein would then subsequently be cleaved from a part of them. (3) RNA synthesis could initiate as in (2), followed by rapid addition of the protein to only a part of the strands. The triphosphate end on the rest of the molecules would then also have to be modified to generate the pUp end of the polyribosomal RNA.

The latter two possibilities seem unlikely. They require that a pyrimidine triphosphate should be the initiating nucleotide. But, virtually all known RNA synthetic systems initiate with purine triphosphates¹⁶. Furthermore, possibility (3) requires that an enzyme cleave off the two 5'-terminal phosphates; in other systems where triphosphates are initiators the mature molecule retains either a di- or triphosphate terminus. Therefore we believe that the present evidence strongly favours possibility (1) but until direct biochemical proof of the model is provided the other alternatives must be considered possible.

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reviews

Botanical dimension in Darwin's thinking R. H. Richens

Darwin and His Flowers: The Key to Natural Selection. By Mea Allan. Pp. 318. (Faber and Faber: London, 1977.) £6.95.

It remains obscure why evolution became scientifically respectable just when it did. Heritable variation at the varietal level was well known and accepted by nurserymen, animal breeders and biologists. The notion of macroevolutionary change was also in the air, as purveyed by Lamarck, Chambers and Charles Darwin's grandfather Erasmus, although less scientifically respectable. Malthus' essay had been widely read. It was not even the case that a vast body of cumulative evidence had to be assembled to vindicate the notion of macroevolution, and it seems that Wallace only required three days to excoquite the theory that Darwin laboured over for so many years.

Perhaps what had been lacking till 1859 was a protagonist in the right club, namely the small group of biologists accepted by themselves and by the general public as representing the forefront of validated scientific thinking. Neither Erasmus Darwin, Chambers nor Wallace were in this club. Charles Darwin, in spite of his lack of formal training, was.

From the point of view of the history of science, then, it becomes more relevant to enquire what led Darwin to sponsor evolution than to ask at what stage the evidence pointed that way. Miss Allan now provides us with some new perspectives. In the course of a very well illustrated account of Darwin's botanical activities, she shows how greatly Darwin was taken up with plants, even though his formal botanical qualifications were thin.

It is clear that Darwin was greatly moved by his encounter with equatorial forest. Possibly, no botanist is most perceptive in his native environment. It is notorious how botany still retains clear evidence of its temperate origins and how potently a small dose of the tropics contributes to theoretical advancement. On the other hand, one can suppose that familiarity with tropical vegetation can be as soporific to its denizens as familiarity with temperate vegetation is to its. It is also clear from Miss Allan's book that

plants attracted Darwin as research material, not only as exhibiting the operation of natural selection, but as doing so without the theoretical complication of "willed" activity.

Miss Allan presents her material in roughly chronological order: Darwin's childhood, Edinburgh and Cambridge, the voyage of the *Beagle*, the *Origin of Species*, his researches on the fertilisation of orchids, climbing plants, plant variation under domestication, insectivorous plants, cross- and self-fertilisation, and heterostyly. Darwin's work is presented very largely in his own terms and in sufficient detail to illustrate his remarkably acute power of observation and experimental ingenuity.

The chief value of Miss Allan's book, beside the pleasure of just looking at it, is the re-orientation that it suggests for the interpretation of Darwin's thought. The botanical dimension has certainly been undervalued in the past. In detail, however,

Miss Allan cannot be our guide. Neither her geology—coprolites as fossil excreta—nor her evolutionary and genetic theory—largely presented in the semi-Lamarckian, pre-Mendelian categories of Darwin's later writings—has been brought up to date.

When it comes to personal interpretation, Darwin becomes unreal; the man has become a scientific hero figure with the inevitable distortions that this produces. Her remarks on Darwin's "illness" are trivial. But one imagines that Miss Allan makes no pretensions in these areas. What she has done, and done well, is to present a vivid picture of the role of plants in Darwin's scientific life, and it was Darwin himself who wrote that "a traveller should be a Botanist, for in all views plants form the chief embellishment". □

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Continuing Newtonian disputes

The Correspondence of Isaac Newton. Volume 6: 1713–17–18. Edited by Rupert A. Hall and Laura Tilling. Pp. xxxviii+499. (Cambridge University: Cambridge and London, 1976. Published for the Royal Society.) £25.

THE core of this sixth volume of the Newton correspondence is made up of a collection of letters and documents relating to the controversy between Newton and Leibniz over the calculus, the commencement of which was noticed in the previous volume. Only a few of these letters and documents are from Newton himself, although some of these are of great interest, such as the hitherto unpublished No. 1053a. On the British side the most common correspondent is Newton's indefatigable champion John Keill, and on the continental side it is Leibniz himself and Johann Bernoulli, with the Italian Conti—eventually a proselyte to the Newtonian camp—acting as intermediary. The editors speculate how Newton might have spent his time if he had not been involved in this controversy with Leibniz. They might equally have speculated how differently things

could have turned out if the controversy had never arisen in the first place, or at any rate had not become envenomed up to the point that British mathematicians effectively cut themselves off for more than a century from the main stream of continental mathematics. It was a strange nemesis that Newton, who by his work in optics, and in the physical, astronomical and philosophical aspects of the *Principia*, had made so vast a contribution both to scientific progress and scientific attitudes in the eighteenth century, and had raised the prestige of British science so high, should have exerted through his mathematical attitudes and techniques so retrograde an influence on British mathematics and mathematical physics in the same century.

This volume witnesses the *dénouement* of the longstanding Newton/Flamsteed controversy in which Flamsteed finally had the better of Newton, largely it seems as a result of the eclipse of certain of Newton's supporters after the accession of George I in 1715. Flamsteed, who now had friends at court, greeted this change of power with glee (letter No. 1151)

and rightly anticipated that it would transform his relations with Newton and the Royal Society over the question of the contentious *Historia Coelestis*. In the event Newton and the referees were forced to call off their campaign against Flamsteed to whom the remaining copies of the *Historia Coelestis* were returned—mostly for burning—together with the manuscripts of the observations on which the work itself had been based, and which Flamsteed then used to prepare his own version of the *Historia Coelestis*, published by his wife some years after his death.

A surprising feature is the absence of any letters between Newton and Clarke relating to the latter's famous controversy with Leibniz although this fell entirely within the span of the present volume. As the editors suggest, this could simply have resulted from Clarke's intimacy with Newton and the fact that they both lived in London, although it should not be assumed that Newton played the same sort of role in the Clarke/Leibniz controversy, as in the controversy over the calculus.

Cotes' letter of 22 December 1713 to Newton (No. 1029) marks the dismal end to the magnificent correspondence relating to the preparation and publication of the second edition of the *Principia*. Whatever the reason, resentment at Newton's failure to thank him

for his vast unpaid labours on the *Principia*, or increasing ill health—he was to die young in June 1716—Cotes coldly rejected all the corrigenda and addenda suggested by Newton in a previous letter. As for some 20 additional errata listed by Newton in the same letter, Cotes had noticed some of them for himself, but had forebore to enter them in his own list of errata lest he should appear “too diligent in trifles”. He ends his letter with a final implicit reproof by pointing out that he had himself silently corrected some hundreds of errors without troubling to acquaint Newton of their existence.

The standard of editing maintains the high level of previous volumes and there is an excellent introduction including a very helpful survey of the controversy over the calculus. An unfortunate omission, however, is any reference in the page opposite the list of contents to the late J. F. Scott's editorship of Volume 4 of the Newton correspondence. Nor do all the footnotes relating to mathematical questions have that luminous clarity which one is entitled to expect. This I found particularly true of note 1 to Newton's notes on Leibniz's ‘Tentamen’ (letter 1069a). This work of Leibniz certainly provides good justification for Voltaire's quip that “Leibniz is come to confuse the sciences” but unfortunately the footnote itself compounds this confusion. Thus it is misleading to say that the theory of Leibniz is “congruent mathematically with that of Newton in the *Principia*” unless by congruent is meant that Newton and Leibniz arrived by entirely different mathematical methods at the same inverse square result for the radial force for motion in an ellipse. Nor is it entirely true that the difference between the two treatments “lies in the *physical* interpretations of Newton and Leibniz”. For although it is true that Leibniz posits a miraculous *ad hoc* ‘harmonic’ vortex to account for the circulatory motion of a planet in conformity with Kepler's second law, nevertheless a careful examination shows that this vortex plays no part whatsoever in the actual mathematical argument in which the one physical factor is the assumption that the force directed towards the focus will be measured—for a given time interval—by the deviation between the actual curved path and the inertial path along the tangent or approximating cord. It is extraordinary that Leibniz should have hit on this notion—one of the most profound physical insights in the *Principia*—before reading the *Principia* itself.

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Illuminating mental darkness

Light and Life. By Lars Olof Björn. (Hodder and Stoughton: London, Sydney, Auckland and Toronto, 1976.) Hardback £3.45; paperback £1.75.

As its title implies, this small book seeks to cover a vast field. All situations where light interacts with living organisms provide a subject for discussion in this book. It begins very naturally with photosynthesis and then moves into the animal kingdom for such topics as bioluminescence and vision. Photoperiodism and spatial orientation with respect to light, influence both animals and plants. There remain some essentially anthropocentric subjects—namely, suntan, artificial illumination and photochemical smog.

The book is based on the original Swedish edition, but some of the later chapters are new additions. It has been well translated and reads very freely. The level is about that of *Scientific American* and, like that journal, it relies heavily on simplified diagrams for the communication of information. Technical detail and jargon are avoided and the emphasis is essentially on readability. This, coupled with its accurate, full and up-to-date content, make it the sort of book one can pick up and read cover to cover. It will appeal most especially to sixth-formers and first-year undergraduates but should, and undoubtedly will, also be read by more advanced students and researchers.

When a small book attempts to cover so much it is not difficult to find gaps. A surprising one is the potential of solar energy for space and water heating and energy farming. One could also criticise the inevitable shallowness of parts of the text. It is not a work of reference to which one might resort for a detailed exposition of, say, photorespiration in C-3 plants or phototaxis in *Euglena*. But at least such topics are mentioned and the basis of their interest and importance is adequately conveyed. The provision of a carefully selected further reading list amply covers the needs of the student whose interest has been fired and who needs to delve deeper. Like the Sun god, Aton, to whom this book is dedicated, Professor Björn should succeed in illuminating the mental darkness of many.

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Computer image processing

Digital Image Restoration. By H. C. Andrews and B. R. Hunt. Pp. xviii+238.) Prentice-Hall International: Englewood Cliffs, New Jersey, and Hemel-Hempstead, UK, 1977.) £19.95; \$31.75. *Digital Picture Analysis.* Edited by A. Rosenfeld. Pp. xiii+351. (Springer: Berlin and New York, 1976.) DM72; \$29.60. *Image Science Mathematics.* Edited by C. O. Wilde and E. Barrett. (Western Periodicals: North Hollywood, California, 1976.) \$37.50.

THESE three volumes—a textbook, a set of essays ranging from high energy physics to cytology, and a collection of contributions to a highly specialised symposium—give a vivid idea of what is happening in computer image processing and of the directions that the subject is likely to take in the near future, although the very new use of computers (D. H. Schaeffer and J. P. Strong, *Proc. IEEE*, **65**, 129–138, 1977) are not discussed.

The textbook, by Andrews and Hunt, is an extremely useful account of the present state of digital image processing, from a mathematical standpoint; it is very much more than a revised edition of Andrews' earlier text on a similar subject (*Computer Techniques in Image Processing*, Academic: New York and London, 1970), for the material has developed and the points of emphasis changed so rapidly that a completely new text has been written. It is clear and carefully organised; the numerous pitfalls and still unsolved problems are signposted; the notation is easily readable; and in regions where tempers have tended to fray in the past, the authors remain commendably objective, their assessment of digital against optical restoration being a good example. The book is divided into three parts: an introduction, covering image formation, recording and models; two chapters on degradations and determination of parameters characterising these; and four chapters on restoration, covering noniterative methods that use Fourier space, the numerous filters generated by a linear algebraic approach and finally, non-linear algebraic methods. *Digital Image Restoration* has only one direct rival: *Digital Picture Processing* by Rosenfeld and Kak (Academic: New York and London, 1976; for review, see *Nature*, **264**, 142, 1976). Both are excellent and the overlap is less than it might seem from a superficial comparison, for the authors' interests are rather different even though much common ground is covered.

Digital Picture Analysis contains five long contributions: automatic, remote sensor image processing by Haralick; radiographic image analysis by Harlow and Dwyer; image processing in high energy physics by McIlwain; digital picture analysis in cytology by Preston; and picture analysis in character recognition by Ullmann. In contrast to the text by Andrews and Hunt, these essays show clearly the successes and problems of processing real large two-dimensional arrays in very different fields. Pattern recognition, in particular texture analysis, is as important here as image restoration; and inevitably very similar problems occur throughout the various applications. It was an excellent idea to bring this material from such different branches of applied science together in a single volume as the subject is still sufficiently young for extensive cross-fertilisation to be taking place. The essays are of a high standard and are written to be comprehensible to a non-specialist audience.

Finally, we turn to the contributions to the symposium on image science mathematics, held in California in

November, 1976. The tone here is very different from that of the other two books under review, for the reader is expected to be conversant with "the story so far", and now ready to read on. There are papers on non-linear restoration, in particular by Frieden on the maximum entropy technique; there are new results on the Karhunen-Loève transform by Jain; Reed *et al.* pursue their work on transforms over finite fields; Krezmar explores the consequences of the lack of a two-dimensional fundamental theorem of algebra; Hunt surveys the (severe) problems of non-linear restoration; Keng and Fu discuss a syntax-directed method of analysing Landsat images; and there are many others. In short, these Proceedings, offset from authors' typescripts, contain much exciting and up-to-date material but they are not for the novice; and furthermore, fuller accounts of the same work are now appearing in the appropriate journals.

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Comparative immunology

Comparative Immunology. Edited by J. J. Marchalonis. (Blackwell Scientific: Oxford and London, 1976.) £15.50.

THE past 15 years have seen a surge of interest in developmental immunobiology. *Comparative Immunology* provides a timely review of the extensive list of research papers in this field and gives insight into phylogenetic and ontogenetic studies currently in progress in the authors' laboratories. Contributions from more than 20 expert immunologists fit together well to make this a really first-rate, readily-readable review.

As a preface to the chapters on phylogeny of immunity, Nossal outlines the cellular, molecular and genetic basis of the complex immune system found in mammals, and Rosser, drawing attention to evolutionary principles, suggests that lower organisms may well have evolved different forms of defence. This suggestion is, in part, borne out by the chapters that follow, on the evolution of cellular immunity. Thus although Cooper presents evidence that all invertebrates, even the Protozoa, can distinguish self from non-self, and postulates the existence of primordial cell-mediated immunity in annelids and echinoderms, Jenkin concludes that recognition by invertebrate phagocytes is based on a system of serum proteins which, although analogous in function,

differ in structure from the immunoglobulins that are unique to the vertebrates. Insight into the physico-chemical and biological properties of non-mammalian immunoglobulins can be gained from later chapters.

Ontogenetic aspects of cellular and humoral immunity in amphibians, reptiles, birds and mammals are examined in some detail. Rowlands describes immune responses of prototherian and metatherian mammals and discusses how these studies are relevant to the study of immune development. Du Pasquier's elegant work reveals that amphibians are proving invaluable in elucidating the mechanism of tolerance, the origin of antibody diversity, the role of the thymus and the nature of the T-cell receptor. Furthermore, Cohen illustrates that "Linnaeus' loathsome amphibians" possess an unusual array of immunological phenomena, while Balls and Ruben make central use of these poikilotherms in excellent reviews on the phylogeny of neoplasia and immune reactions to tumours, and on the nature of cell co-operation.

The book should prove invaluable to all immunobiologists wishing to learn in depth about the evolution of immunity in the entire animal kingdom and of the advantages that can be gained by using simple model systems to study central issues in immunology.

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Water and aqueous solutions

Liquids and Solutions: Structures and Dynamics. By P. Kruus. Pp. 592. (Marcel Dekker: New York and Basel, 1977.) SFRs. 145.

THIS book is based on a graduate course and seminars, and is intended for physical chemists and people with related interests—for example, engineers and biologists. Certain topics are deliberately omitted—for example, glasses, melts, quantum liquids, liquid metals, and liquid crystals. A feature is the considerable attention given to water and aqueous solutions. These topics are of the greatest interest to other scientists but are often neglected because water (or an aqueous solution) is extremely difficult to handle theoretically. The author does not try to deal with any topic in depth but has produced what amounts to a very interesting series of review articles with a very large number of appropriate references. These will certainly be valuable both to specialist research workers, and to workers in related fields, for many years to come. One is struck both by the author's vast reading, and by his ability to summarise a particular field and put it into proper relation with other fields.

The book is divided into three parts. Part A deals with theoretical approaches: what are the forces between molecules or ions and how do we allow for their effects? Model treatments, distribution function theory and the theory of electrolytes are all summarised with appropriate references, but the reader is always left wanting more. Two chapters on transport properties and molecular motions can only be described as sketchy.

Part B is concerned with experimental studies. We can distinguish between what we can deduce from the equilibrium properties and transport coefficients (chapters 8 and 9), what we can deduce by bombarding the liquid with neutrons or by imparting various forms of energy to it (chapters 10 and 11, and 14–16), and what we can learn by using a typical molecule as a probe in magnetic resonance and spectroscopic studies (chapters 12 and 13). Each chapter has some specific examples of the application of each method. A considerable amount of very interesting material is given, but one would have welcomed a further chapter explaining exactly what sort of information can legitimately be expected from each method. For example, it may seem elementary to the author that because of wavelength considerations, ultrasonic and light-scattering studies tell us nothing about

the distribution function but a great deal about critical fluctuations; but such information would be welcome to some of his likely readers.

Part C gives very brief accounts of thermodynamics statistical mechanics, electromagnetism and quantum mechanics. This is the weakest feature of the book. Although over 70 pages are used, these chapters are no substitute for a proper textbook, as the author himself recognises. If these topics are to be treated at all, why not include hydrodynamics also? I would like to have seen these pages used instead in expanding the excellent parts A and B, some chapters of which

have quite clearly had to be unduly compressed.

Despite this tactical error, I adhere to my opinion that the author has done a good job in summarising what is now a considerable body of knowledge and that the result will be helpful to research workers. The emphasis on water and aqueous solutions is particularly to be commended at the present time, much of the material given being hard to find elsewhere.

H. N. V. Temperley

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Complexities of intelligence

Intelligence: Nature, Determinants and Consequences. By E. B. Brody and N. Brody. Pp. x+241. (Academic: New York and London, 1977.) \$12.50; £8.85.

INTELLIGENCE TESTING has been with us for some seventy years, and the results used—and sometimes misused—for important social and educational purposes during much of this period. From the start, at least two irreconcilable lines of thought about intelligence test scores can be traced. The first was at the outset peculiarly British, initiated by Galton and developed by Spearman, suggesting that there exists a central, inherited general intellectual capacity, fixed and unalterable, and directly measurable by tests. The second, which arose from the work of Binet in Paris, stressed the potentially plastic nature of intellectual functions.

Today, the questions of plasticity of intelligence, the degree of its heritability both within and between groups and races, remain central and controversial. The debate has been sharpened by Jensen's article (1969) which suggested a strong inherited component in the well-attested average negro-white IQ differences, and by revelations in 1974 by Jensen and by others, that some of the major post-War data of Sir Cyril Burt, the arch-hereditarian, were worthless.

The book by Brody and Brody addresses itself to problems such as these, sketching initially a little of the historical background, considering the work of Cattell and Guilford on the structure of the intellect, and outlining the quantitative characteristics of global indices. The relationship between test scores and achievement is reviewed and the respective roles of genetic, biological and social environmental factors as determinants are considered. A

critical chapter is devoted to the problems of racial differences, birth order and family size, and the volume concludes with a review of the use of tests in the educational system, in selection and in clinical assessment.

Like many new American books in this field, this volume reflects the radical change which has taken place in recent thinking on what intelligence tests measure, their social and educational applications, and the constellation of variables which affect their results. It is up-to-date, clearly written and indicative of an unusual width and depth of knowledge. Surprisingly, however, it is weak in the section on stability and change in individual intelligence during the lifespan, considering in detail only one major longitudinal study; indeed, it underestimates the amount of relative change occurring in children reared in fairly constant environments. It also pays too little attention to the many careful studies on larger IQ changes following major environmental shifts. Yet the authors are rightly doubtful of "critical period" hypotheses which suggest that only early interventions may be rewarding for the disadvantaged. They fail, however, to spell out in detail the implications for successful interventions, in particular duration and totality.

Here and there, one can fault the authors' statements: for example, the results of the important Dutch Famine Study are open to alternative explanations than the one they favour, and they do not reveal any awareness of this controversy. The omission of an author index and the sketchiness of the subject index are also surprising. All in all, however, this book achieves its aim—a balanced account of the major issues. It will be valuable to those who seek an introduction to understanding these complexities.

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obituary

Pehr Edman

DR PEHR EDMAN, FRS, died on 19 March 1977, in Munich, at the age of 60. In an eventful and distinguished scientific career on three continents, he was undeniably one of the most influential figures in advancing our understanding of the chemical structure of proteins.

Pehr Edman was born in Stockholm on 14 April 1916. Though his undergraduate training was in medicine, very early on he was attracted to the field of biological chemistry, then in its infancy, and in the 1940s carried out (with U. S. Von Euler and E. Jorpes) some of the earliest work on the chemical characterisation of angiotensin. He later spent a year at the Rockefeller Institute in the laboratory of John H. Northrop, studying the effects of tyrosinase on several proteolytic enzymes.

In retrospect it is difficult to envisage the primitive state of methods for peptide analysis at that time. An amino acid analysis of a protein was a major undertaking; sequence analysis of even a small peptide was a chemical *tour de force*. Pehr Edman clearly recognised the need for better analytical techniques for proteins, and was to devote almost his whole scientific career to developing them.

He approached this task with thoroughness and attention to detail illuminated by a clarity of thought characteristic of the man. His critical insight was to see that earlier attempts at an end group procedure for peptides by Max Bergmann and Emil Abderhalden could be converted into a sequential degradation procedure by simply substituting sulphur for oxygen in the reagent used. This led to one of the most important, (and most often cited) papers in modern biological literature: 'A Method for the Determination of the Amino Acid Sequence in Peptides' published in *Acta Chemica Scandinavica* in 1950.

From this point Edman's international reputation was established, and he could easily have taken the method as it then was and applied it to structural studies on proteins of biological interest. High honours would have surely and rapidly followed, as they did to others. (Sanger, earlier, and Moore, Stein, Anfinsen and Edelman, later, received Nobel Prizes for their studies on protein structure and function). Pehr Edman typically took a

more difficult road. He set out to perfect the sequential degradation method, a task that was to occupy the rest of his life. The way he went about this is in itself a fascinating story. Suffice it to say that in 1967, with a talented colleague in Melbourne, G. Begg, he published in the first issue of the *European Journal of Biochemistry* an article describing an instrument, the 'sequenator,' for the automatic determination of sequences in proteins. This instrument is now produced commercially and is in use in many laboratories around the world. Its availability has vastly accelerated progress in molecular biology. For this work Pehr Edman was awarded in 1971 the Commonwealth Britannia Award, and the rare Berzelius Gold Medal (the eighth awarded this century). In 1974 he was elected to the Royal Society of London.

At a personal level Pehr Edman was an interesting and complex man. Close or intimate relationships were rarely established even by those who worked in the same field or who followed his methods closely. I was privileged to be one of a small number of his students and close associates. These included John Sjoquist, Birger and Margareta Blombäck, Agnes Henschen (later his wife), Frank Morgan, and of course his colleague for fifteen years, Geoffrey Begg. Pehr rarely spoke of personal matters, but it seems that the end of his first marriage in divorce and some disappointments in local recognition and advancement in Sweden may have contributed to his decision to move to Melbourne in 1957 as Director of St Vincent's Hospital School of Medical Research.

Initially this must have been more a self-imposed exile than a positive career decision. The laboratories at St Vincent's prior to his arrival were non-existent. Apart from the late Professor John Hayden and one or two others, the staff at St Vincent's in 1957 had little idea of the real potential of Edman's work or of his outstanding scientific abilities. As his work had no obvious immediate clinical relevance he was largely ignored by the hospital. Pehr Edman's reserved, not to say withdrawn, personality contributed to this. The end result was that he worked for many years in virtual isolation both from St Vincent's Hospital and with a few exceptions from the Australian scientific community. He gained at least the local reputation of being un-

sociable and difficult. On the contrary, he was, I believe, a rather shy person who simply did not have the sort of extroverted nature required to make his presence felt in the scientific wilderness of Australia in which he found himself.

To his close associates and friends, and to scientific visitors to his laboratory he was a totally different man—warm, helpful and unfailingly courteous. However, he was uncompromising in his attitude to anything he regarded as scientifically unsound. In his own work he was overwhelmingly competent. He enjoyed working at the bench, designed and made his own instruments and was an excellent glass-blower. In the European tradition, the laboratory was run on disciplined lines. No short-cuts or shoddy techniques were tolerated. He put great emphasis on self-reliance and careful use of equipment. At least in the early days in Australia he refused to buy the commercially produced micropipettes of Lang-Levy design. Each was made and calibrated in the laboratory and kept with scrupulous care. Chemicals were never used straight out of the bottle; before each experiment began he would personally carry out a careful redistillation or recrystallisation of the required reagents. His approach was totally professional.

Pehr Edman was a man of cultivation, widely read, interested in music and the arts, and a gourmet cook. At morning and afternoon tea breaks at work his ritual was to sit down, light one of his favourite thin cigars and slowly drink a cup of very strong freshly ground coffee. The conversation, usually unrelated to science, reflected his wide interests and cosmopolitan attitudes.

Though at first he must have felt the scientific and personal isolation in Australia, Pehr grew to love his country of adoption and later became an Australian citizen. He particularly enjoyed fishing and bird-watching, and few scientific visitors from overseas escaped a day-long excursion to Sherbrooke Forest, near Melbourne, to catch a glimpse of the elusive Australian lyre bird.

In 1972 he left Australia for Munich to be head of a new protein chemistry unit at the Max-Planck-Institut für Biochemie at Martinsried. Unhappily he did not long survive in this new venture. He leaves a wife, Dr Agnes

Henschen-Edman, and two young children, Carl and Helena.

Though his career was tragically cut short, he succeeded brilliantly in the task he set himself. One wonders what scientific problem would not have succumbed to Pehr Edman's creative and single-minded endeavours.

H. D. Niall

C. J. Smithells

DR COLIN J. SMITHELLS who died on 25 March 1977, held a variety of posts in the metallurgical field since he graduated in chemistry at Leeds University in 1914.

Immediately after graduation he joined the Army in which he served with distinction throughout the 1914-18 war reaching the rank of Major in the Gloster Regiment, gaining the Military Cross and being mentioned in dispatches.

In 1919 he joined the research staff of the General Electric Company at the new laboratories being set up by Sir Clifford Patterson at Wembley. There he was primarily responsible for the production and fabrication (by the new metallurgical techniques such as powder metallurgy) of the less well-known metals—with particular reference to tungsten and the manufacture of lamp filaments, receiving valves etc. His book *Tungsten* continues to hold the field and other books, such as *Gases in Metals* have established with authority the existing state of knowledge at the time they were written.

He was awarded the degree of DSc. by Leeds University in 1921. While still at GEC in 1937 he developed a Heavy Alloy (of tungsten nickel and copper) particulars of which were first published in *Nature* in 1937.

In 1938 he left the GEC to become General Manager of Lodge Plugs of Rugby where he was concerned with the use of platinum alloys until 1944 when he was appointed Director of Research of the British Aluminium Company. Here he was responsible for the setting up of the Company's new and now well-known Research Laboratories at Chalfont Park in Buckinghamshire.

In 1956 he retired from Chalfont Park to become Managing Director of Magnesium Elektron Limited, which post he held until he finally retired in 1963.

Throughout his working life he devoted time generously to the work of organisations which aimed to benefit the industry and profession of metallurgy. He was, in 1945, a Founder Fellow of the Institution of Metallurgists, of which he was President in 1951-52. In the following year he was President of the Institute of Metals and

throughout gave much time to the work of government committees and industrial bodies such as the British Non-Ferrous Metals Research Association and the Aluminium Development Association. In 1949 he edited the first issue of the *Metals Reference Book*, a comprehensive collection of data which he saw through several new editions.

He was outstanding in the profession for his charm of manner and clarity of thought and speech and will be a great loss. He is survived by his wife Mary, two sons and three daughters.

G. L. Bailey

R. N. Singh

PROFESSOR R. N. SINGH, formerly Head of the Botany Department of Banaras Hindu University, died from heart failure on March 9, 1977. He was internationally recognised as an authority on phycology, to which he contributed much original observation and in which he initiated new lines of research. Especially, he was distinguished for his work on nitrogen fixation by blue-green algae in rice fields, making contributions both to botanical knowledge and practical agriculture. Besides isolating and characterising various nitrogen-fixing species, he was one of the first to study the paddy field as an ecosystem. His use of the simple expedient of enclosing areas of alkaline "Usar" lands, so that ponds form in the rainy season and prolific growth of blue-green algae is encouraged, is a method of reclamation the full potential of which has not yet been realised. This and other work is described in his book, *Role of blue-green algae in nitrogen economy of Indian agriculture* (1961). He also carried out research on the taxonomy, morphology, physiology, radiation biology, genetics and virology of blue-green algae. In the course of this work he produced many mutant strains of blue-green algae, which have interesting implications for our understanding of morphogenesis in these organisms.

R. N. Singh was born on August 2, 1915, in the Deoria district of Uttar Pradesh and was educated at the U.P. College, Varanasi and Even Christian College, Allahabad. He obtained the M.Sc. degree in 1938 at the Banaras Hindu University while working under Professor Y. Bharadwaja. In 1946 he was awarded the D.Sc. of the same University. He worked in several famous laboratories abroad including the Department of Botany, University College London, the Department of Biochemistry, University of Wisconsin (where he participated in the preparation of the first cell-free extracts active in nitrogen fixation), and the Plant Research Laboratory, Michigan State University. He travelled widely and

was a familiar figure at international conferences on phycological and limnological topics.

Besides being a creative research worker, Professor Singh was an outstanding teacher of plant physiology as well as phycology and many of his students now hold responsible posts in different parts of the world. He established a well equipped phycological laboratory in the Banaras Hindu University where he trained more than thirty Ph.Ds in various branches of phycology. After retirement he occupied himself with writing on recent developments in the study of blue-green algae. It is to be hoped that these nearly completed works will be published in due course. His sudden death is a sad loss to Indian science and phycology generally. He will be remembered with affection and gratitude by his many friends and students.

P. K. Singh
G. E. Fogg

Eli Lilly

ELI LILLY, former President and Chairman of Eli Lilly and Company of Indianapolis died on 24 January at the age of 91. He was a very remarkable man. From the age of 10 he worked with his father Josiah K. Lilly Senior, in the family business and in 1907 qualified as a pharmaceutical chemist from the Philadelphia College of Pharmacy and Science, where his father also had studied. Thereafter he developed an interest, ahead of his time, in efficiency of production methods, becoming superintendent of the manufacturing division in 1909, general superintendent in 1915, and vice-president from 1920 until 1932 when he succeeded his father as president. He guided the company through the difficult Depression years of the early 1930s and despite the economic downturn no employees were laid off. Under his overall guidance the production of insulin for diabetes was developed in the 1920s, the production of liver extract for pernicious anaemia, and of barbiturates, in the 1930s; and after the war production of penicillins and other antibiotics including erythromycin.

Lilly was a great philanthropist. He endowed a memorial laboratory in 1936 at the Philadelphia College of Pharmacy and founded Lilly Endowment, Inc. in 1937 which over the years has given some 250 million dollars to charitable causes. His wide-ranging interests included educational and civic activities, stock-breeding and archaeology. His wife died in 1973 and their daughter in 1970. There are no immediate survivors.

Frank Hartley

announcements

On the Move

S. Sampanthar, Department of Mathematics, University of Salford, to Department of Mathematics, University of Calabar P.M.B. 1115 Calabar, Nigeria on 11 July 1977.

Details of changes of department, sabbaticals, where leave will be taken and so on should be sent to On the Move; there is no charge for this service.

Awards

Charles Earp of Standard Telecommunications Laboratories is the first recipient of the new award, the J. J. Thompson Medal, of the Institution of Electrical Engineers, for his work on communication and radio navigational aids.

W. E. Kliner was the first recipient of the Douglas Bomford Trust Prize from the Institution of Agricultural Engineers for the best technical paper, entitled 'The Field Treatment of Grass for Conservation', presented at the 1976 Annual Conference.

The European Physical Society has awarded the 1977 Hewlett-Packard Europhysics Prize (20,000 S.Fr.) to Professor Walter Eric Spear of the Carnegie Laboratory of Physics at the University of Dundee in Scotland for his work on a special form of semiconductor which is non-crystalline and can be prepared as thin films.

B. Heezen is the recipient of the 1977 Walter H. Bucher Medal for original contributions to the knowledge of the Earth's crust.

P. Richards received the 16th James B. Macelware Award for significant contributions to geophysical sciences.

Appointments

D. A. L. Saunders to the Chair of Architecture at the University of Adelaide, South Australia.

D. J. Cove to Professor of Genetics and Head of Department at Leeds University from 1 January 1978.

Dr R. S. Barnes, to Principal of Queen Elizabeth College from 1 April 1978.

Sir Richard Doll, OBE, Regius Professor of Medicine at the University of Oxford to chairman of the Committee of Management of the Institute of Cancer Research from 1 January 1978.

Professor F. L. Mastaglia, to the Chair of Experimental Neurology, University of Newcastle-upon-Tyne.

Dr M. J. Sewell, to a Personal Professorship in Mathematics at Reading University from 1 June 1977.

Dr R. F. Curtis, to Director of Agricultural Research Council Food Research Institute at Norwich on 1 October 1977.

D. Eccleston, to the Chair of Psychological Medicine from 1 October 1977, and also to Head of the Department of Psychological Medicine for five years from 1 October 1977.

Person to Person

Furnished 3/4-bedroom house, Manchester area, available 1 March 1978 to 28 February 1979. Contact M. Sampanthar, 25 Vaudrey Drive, Cheadle Hulme, Cheshire, UK.

Exchange furnished 5-bedroom house in Gainesville, Florida for similar convenient to Mill Hill, London, for 1 year, September 1977 to August 1978. Rental of London house also considered. Contact D. Duckworth, Box J-266, JHM Health Center, Gainesville, Florida 32610.

Exchange furnished, 7-room house south-east London, end August 1977 to July 1978, for house or apartment in Boston/Cambridge, Massachusetts area. Contact J. A. Patterson, Apt. C511, 77 Pond Avenue, Brookline, Massachusetts 02146.

There will be no charge for this service. Send items (not more than 60 words) to Marcus Dobbs at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

International Commission for protection against environmental mutagens and carcinogens

This commission (ICPEMC) has been established to primarily approach the problems involved in minimising hazards of the increasing variety of mutagenic chemicals to which human populations are exposed. Current knowledge is to be reviewed with the aim of formulating recommendations to serve as bases for regulatory action.

Lady Tata Memorial Trust

The Trustees of the Lady Tata Memorial Trust for research in leukaemia have made the following International Awards for the Academic Year 1977-78. R. de N. Bastos (Spain), L. Gauci (Great Britain), P. Mignatti (Italy) (2nd year awards); H. Jacobs (Great Britain), $\frac{3}{4}$. Ravid (Israel) (New awards); E. Cardoso (Portugal), G. Grosslerner (France) (New awards from April 1977).

Meetings

25-29 July, **International Conference on Solar Building Technology**, London, UK (The Secretariat, International Solar Building Conference, North East London Polytechnic, Forest Road, London, UK).

15-17 August, **Symposium on Prospecting in Areas of Glaciated Terrain**, Helsinki (The Institution of Mining and Metallurgy, 44 Portland Place, London, UK).

2-5 August, **Cryogenic Engineering/Materials Conference**, Boulder, Colorado (Bureau of Conferences and Institutes, Academy 217, 970 Aurora Avenue, University of Colorado at Boulder, Boulder, Colorado 80302, USA).

23-26 August, **5th International Conference on Chemical Thermodynamics**, Ronneby, Sweden (Fifth International Conference on Chemical Thermodynamics, c/o RESO Congress Service, S-105 24 Stockholm, Sweden).

24 August, **19th Canadian High Polymer Forum**, Ottawa (Secretary-Treasurer, 19th Canadian High Polymer Forum, National Research Council of Canada, Division of Chemistry, Montreal Road, Ottawa, Ontario, K1A 0R9, Canada).

28-30 August, **Satellite Symposium to the VI International Meeting of the International Society for Neurochemistry: Endorphins**, Brescia, Italy (F. G. Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

31 August-2 September, **2nd Drag Reduction Conference**, Cambridge, UK (Organising Secretary, 2nd Drag Reduction Conference, BHRA Fluid Engineering, Cranfield, Bedford, UK).

12-16 September, **Symposium on Unconventional Photographic Systems**, Oxford, UK (Royal Photographic Society of Great Britain, 14 South Audley Street, London W1, UK).

14-16 September, **The Oil Industry and Microbial Ecosystems**, Warwick (The Institute of Petroleum, 61 New Cavendish Street, London W1, UK).

Reports and Publications Other countries—May

- Smithsonian Contributions to Zoology, No 247: The Entocytherid Ostracods of North Carolina. By Horton H. Hobbs, Jr., and Daniel J. Peters. Pp. iv + 73. (Washington, DC: Smithsonian Institution Press, 1977. For sale by US Government Printing Office.) [45]
- Bulletin of the Florida State Museum, Biological Sciences, Vol. 20, No. 4: Fossil Sirenians and Desmostyliids from Florida and Elsewhere. By Roy H. Reinhart. Pp. 187-300 \$3.85. Vol. 21, No. 1: Variation and Relationships of Some Hispaniolan Frogs (Lepidactylidae, *Eleutherodactylus*) of the *Ricordi* Group. By Albert Schwartz. Pp. 1-46. \$1.70. Vol. 21, No. 2: The Natural History of the Alabama Map Turtle, *Graptemys pulchra* Baur, in Alabama. By Robert M. Shealy. Pp. 47-111. \$2.10. (Gainesville, Florida: Florida State Museum, University of Florida, 1976.) [45]
- United States Department of the Interior: Geological Survey, Bulletin 1418: Weak-Field Magnetic Susceptibility Anisotropy and Its Dynamic Measurement. By William F. Hanna. Pp. iv + 73. (Washington, DC: US Government Printing Office, 1977.) [55]
- Food and Agriculture Organization of the United Nations. Food and Environment: Reconciling the Demands of Agriculture with Global Conservation. Pp. 44. (Rome: FAO; London: HMSO, 1976.) £1.62. [55]
- Université du Québec. Un Réseau de Personnes—Ressources à Votre Service. Pp. 164. (Québec: Université du Québec, Institut National de la Recherche Scientifique, 1977.) [55]
- Organisation for Economic Co-operation and Development, Energy Production and Environment. Pp. 107. (Paris: OECD; London: HMSO, 1977.) F.22; £2.60; \$5.50. [65]
- OECD Planning Group on Science and Technology for Developing Countries. Report of the Steering Group on Pest Control Under the Conditions of Small Farmer Food Crop Production in Developing Countries. Pp. 83. (Paris: OECD, 1977.) [65]
- Battelle Memorial Institute. The President's Report and Annual Review 1976. Pp. 28. (Columbus, Ohio: Battelle Memorial Institute, 505 King Avenue, 1977.) [95]
- Université du Québec. L'Institut National de la Recherche Scientifique—Descriptive Brochure. Pp. 78. (Québec: Institut National de la Recherche Scientifique, 1977.) [95]
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